POLITÉCNICO DE LEIRIA

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Extraction, characterization and application of phlorotannin enriched extracts from macroalgae ***Fucus spiralis***

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Resumo

Fucus spiralis (*F. spiralis*) é uma alga castanha, abundante em águas frias a temperadas ao longo do hemisfério norte. Estas algas são ricas em compostos bioativos, de entre os quais se destacam os florotaninos que são polifenóis derivados do floroglucinol com importantes propriedades antioxidantes.

Os principais objetivos deste trabalho foram i) avaliar a influência da sazonalidade no conteúdo em compostos fenólicos e capacidade antioxidante de extratos de *F. spiralis* obtidos por meio de extração ecológica usando solventes de grau alimentar; e ii) avaliar o efeito antioxidante da incorporação de um extrato enriquecido em florotaninos em homogeneizados de salmão.

A biomassa de *F. spiralis* colhida nas quatro estações foi inicialmente submetida a uma extração com etanol:água (extratos brutos Et80), seguida de um fracionamento líquido-líquido com solventes orgânicos (fração de n-hexano He; frações aquosas AQ1, AQ2, AQ3 e AQ4; fração de acetato de etilo EA).

Todos os extratos (Et80, He, AQ1, AQ2, AQ3, AQ4 e EA) obtidos para a *F. spiralis* colhida nas quatro estações foram depois avaliados quanto ao conteúdo em compostos fenólicos e capacidade antioxidante. Das quatro estações, os extratos de verão apresentaram teores de polifenóis (TPC) mais elevados e também o maior poder antioxidante férrico redutor (FRAP). A capacidade de redução do radical DPPH (2,2-difenil-1-picril-hidrazil) foi semelhante entre estações. Para todas as estações, a fração EA apresentou maior teor de polifenóis (TPC), e maior poder antioxidante (maior poder redutor férrico (FRAP), e menor concentração necessária para reduzir 50% do composto DPPH).

O extrato EA obtido a partir de alga recolhida no verão foi o que apresentou maior teor em compostos fenólicos e maior poder antioxidante e foi, por esse motivo, utilizado para avaliar o seu potencial como um antioxidante natural na extensão do tempo de vida útil de homogeneizados de salmão, comparando-o ao antioxidante artificial BHT. A incorporação do extrato de alga não mostrou diferenças significativas ao nível do pH e da humidade das amostras ao longo do tempo de armazenamento (21 dias), em comparação com as amostras contendo BHT ou com o controlo. A adição do extrato teve um comportamento semelhante à adição de BHT na diminuição dos teores de deterioração do peixe (diminuição das espécies reativas do ácido tiobarbitúrico TBARs) nos primeiros dias do estudo (até ao dia 11), em comparação com o controlo. Relativamente à alteração da cor, nenhum dos antioxidantes permitiu reduzir a perda de cor, não se tendo verificado

alterações significativas nos parâmetros medidos. Em relação à quantificação de azoto básico volátil total (ABVT), nenhuma diferença significativa foi observada entre os tratamentos.

Este estudo revelou que o teor de polifenóis e o poder antioxidante dos extratos de *F. spiralis* são influenciados pela época de colheita assim como pelos solventes usados na extração. Apesar de promissor ao nível do conteúdo em compostos fenólicos e da capacidade antioxidante, o extrato EA obtido a partir de alga recolhida no Verão não revelou capacidade antioxidante significativa quando aplicado em homogeneizados de salmão.

Palavras-chave: *Fucus spiralis*, atividade antioxidante, polifenóis, florotaninos.

Abstract

Fucus spiralis (*F. spiralis*) is a brown macroalgae, abundant in cold-temperate waters along the northern hemisphere. They are rich in bioactive compounds, including phlorotannins that are polyphenols derived from phloroglucinol and display important antioxidant properties.

The main objectives of this work were i) the evaluation of the seasonal variability of the content in phenolic compounds and antioxidant power of *F. spiralis* extracts obtained through eco-friendly extraction using food grade solvents; and ii) the evaluation of the antioxidant effect of the incorporation of a phlorotannins-enriched extract in salmon homogenates.

The *F. spiralis* biomass collected in the four seasons were submitted to an extraction with ethanol:water (crude extracts Et80), followed by a liquid-liquid fractionation with organic solvents (fraction He from n-hexane; aqueous fractions AQ1, AQ2, AQ3 and AQ4; ethyl acetate fraction EA).

All the extracts (Et80, He, AQ1, AQ2, AQ3, AQ4 and EA) obtained for *F. spiralis* of the four seasons were then evaluated for their antioxidant capacity and total phenolic compounds. Summer extracts presented the higher contents in polyphenols (TPC) as well as the higher ferric reducing antioxidant power (FRAP), when compared to the samples from other seasons. The reductive percentage of DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) compound was similar between seasons. For all seasons, EA extract showed the higher polyphenol content (TPC), and the higher antioxidant capacity (higher ferric reducing power (FRAP) and lower concentration needed to reduce 50% of DPPH compound).

The EA summer extract presented the higher content in phenolic compounds and the higher antioxidant power. For that reason, it was used to evaluate its potential as a natural antioxidant to extend the shelf-life of salmon homogenates, while comparing it to artificial antioxidant BHT. The addition of the algal extract did not show significant differences in the maintenance of pH and humidity of the samples throughout testing time (21 days) when compared to the samples with artificial antioxidant BHT and the control. The extract had a similar behavior to the artificial antioxidant BHT regarding the diminishing of deterioration compounds in fish (decrease of thiobarbituric acid reactive species TBARs) on the first days of the study (until day 11) when compared to the control. Relative to color alteration, none of the antioxidants decreased color loss, as no significant changes were observed in the

measured parameters. Regarding the quantification of total volatile basic nitrogen (TVB-N), no significant difference was observed among treatments.

This study revealed that the content of polyphenols and antioxidant power of the *F. spiralis* extracts are influenced by the time of harvest as well as by the solvents used for its extraction. Although promising in terms of the content of phenolic compounds and in terms of antioxidant capacity, the EA summer extract did not reveal significant antioxidant capacity when applied to salmon homogenates.

Keywords: *Fucus spiralis*, antioxidant activity, polyphenols, phlorotannins.

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Abbreviations List

AQ1: Aqueous fraction 1

AQ2: Aqueous fraction 2

AQ3: Aqueous fraction 3

AQ4: Aqueous fraction 4

BHT: Butylated hydroxytoluene

CPE: Centrifugal Partition Extraction

DPPH: 2,2-diphenyl-1-picryl-hydrazyl

EA: Ethyl Acetate fraction

EAE: Enzyme-Assisted Extraction

AAE: Ascorbic Acid Equivalent

GAE: Gallic Acid Equivalent

EDTA: Ethylenediaminetetraacetic acid

Et80: Crude ethanol-water (80:20) extract

EtOH: Ethyl alcohol

FRAP: Ferric Reducing Antioxidant Power Assay

G1-FS: Salmon patties with phlorotannins-enriched *Fucus spiralis* extract (0.01%)

G2-BHT: Salmon patties with synthetic antioxidant BHT (0.01%)

G3-Ct: Salmon patties without antioxidant incorporation (control)

He: n-Hexane fraction

IC_{50}: Half maximal inhibitory concentration

MA: Malonaldehyde

MAE: Microwave-Assisted Extraction

PLE: Pressurized Liquid Extraction

PUFA's: Polyunsaturated fatty acids

ROS: Reactive Oxygen Species

SFE: Supercritical Fluid Extraction

TEP: 1,1,3,3-tetraetoxipropane

TBARS: Thiobarbituric acid reactive substances

TVB-N: Total volatile base nitrogen

ABVT: Total Volatile basic nitrogen

TPC: Total Phenolic Content

TPTZ: 2,4,6-Tri(2-pyridyl)-s-triazine

UAE: Ultrasound Assisted Extraction

1. Introduction

1.1. Macroalgae

Macroalgae are a group of aquatic organisms that belong to the *Plantae* kingdom (Marques, 2017); with autotrophic metabolism that use chlorophyll a as the primary photosynthetic pigment (Dreckmann et al., 2013). They are very simple organisms composed of one cell or grouped together in colonies or as organisms with many cells, sometimes collaborating together as simple tissues (Kim, 2012).

The body of macroalgae is known as thallus, which is the vegetative body of the algae that has no differentiation of roots, stems and leaves, unlike high level plants (Melsasail et al., 2018). They also lack lignin, since they do not need stiffness or rigidity in the water (Marques, 2017). Depending on the secondary pigments, they are classified into three classes: Rhodophyceae (red), Chlorophyceae (green) and Phaeophyceae (brown) (Pimentel et al., 2017).

Red algae known as Rhodophyceae present a double membrane in the chloroplast, chlorophyll a, as main component and chlorophyll d, in minor proportion (Estevez, 2003). The red colour is derived from Chlorophyll a, phycoerythrin and phycocyanin. There are 4000-6000 species of red algae in over 600 genera and most of them exist in tropical marine environments (Marques, 2017).

Chlorophyceae, also known as green algae, generally have predominantly green chlorophyll pigments; they also contain subordinate carotenoid and xanthophyll pigments and are the ancestral relatives of vascular plants. Green algae are always present on tropical coral reefs and lagoon floors, often intermixed among sea grass shoots (Littler & Littler, 2013).

Brown algae, classified as Phaeophyceae, contain chlorophyll a and c as their main photosynthetic pigment, as well as beta-carotene and other xanthophylls (Marques, 2017). They contain large quantities of the brown pigment fucoxanthin and have layered cellulose walls with an outer layer of alginic acid and fucoidin. There are about 2000 species of brown macroalgae and they are almost exclusively marine algae (Littler & Littler, 2013).

1.2. Brown macroalgae

Phaeophyceae represent a large and heterogeneous class with more than 2000 species that have been described within 285 genera, 50 families and 19 orders (Wehr, 2016). They inhabit predominantly in the marine environment and are found in the supra, meso and infralittoral zones, especially in the polar, boreal and temperate regions.

The size of their stalks varies from microscopic to gigantic (kelps) and can be filamentous, pseudoparenchymal and parenchymatous. Filamentous stalks consist of individual filaments, or filament aggregates to give robustness to the body. The pseudoparenchymatous is composed of filament aggregates but without true tissue, while the parenchymatous already develops by cell division in several planes. They present tricotalic, diffuse, apical and intercalar growth (Da Silva, 2018).

Brown algae have cell walls composed of alginates, sulfated fucans, and cellulose, in average proportions of 3:1:1. The walls may also be supplemented with phlorotannins (phenolic compounds), that accumulate in cytoplasmic inclusions known as physodes and lesser quantities of proteins (Wehr, 2016). Phaeopyceae contain the pigments chlorophyll a, c1 and c2, as well as fucoxanthins, which are the cause of the brownish colour of the macroalgae; the composition of carotenes is limited almost exclusively to β-carotene (Kornprobst, 2014).

Its reserve materials, or substances stored in the algal cells as food materials, are laminarine, a water-soluble β-1,3-glucan-linked polysaccharide (Gupta & Abu-Ghannam, 2011) and mannitol, a six-carbon open chain polyalcohol that represents 20-30% of the dry weight of brown algae (Da Silva, 2018).

1.2.1. ***Fucus spiralis***

Fucus is an abundant and widely distributed genus of brown, perennial and edible macroalgae, and occupies the cold-temperate waters from the littoral and sublittoral regions along the rocky shorelines of the northern hemisphere. The genus is comprised of 66 species characterized by a greenish brown trisected thallus, a small stipe and flattened dichotomously-branched blades with terminal receptacles that swell during the reproductive season. The blades usually have a central-thickened area called midrib and, in some species, air bladders can be found (Catarino et al., 2018).

The species from this genus are among the brown algae that accumulate the highest amounts of phlorotannins, with almost 12% of dry weight; however, their concentration is dependent on the season, solar exposure, salinity and geographical origin. The phlorotannins from this genus have promising antioxidant, anti-inflammatory and antitumor activities (Catarino et al., 2019).

F. spiralis is the uppermost species of the genus *Fucus* that occurs on the shores. It has a slightly greenish brown colour, similar to *Fucus vesiculosus*, and grows up to 30 cm of length. Its frond lacks bladders and does not have a serrated edge, being sometimes spiralled. Reproductive organs form swollen and rounded extremities in the branches, usually coupled (Fig. 1.1), (The Seaweed Site, 2014). It's found on the Atlantic coasts of Europe and North America (Pinteus et al., 2017), as seen in figure 1.2.

Kingdom	Chromista
Phylum	Ochrophyta
Class	Phaeophyceae
Order	Fucales
Family	Fucaceae
Genus	*Fucus* L.

Figure 1.1: Detail of *Fucus spiralis* fronds and systematic classification of the specie (White, 2008 & GBIF Secretariat, 2019).

Figure 1.2: Distribution of *Fucus spiralis* (White, 2008).

It is eaten as a delicacy in some locations since it is very rich in fiber (63% dry weight) and contains minerals such as Na, Mg, Ca and K. Additionally, lipid content is low (5.2%) and it is also rich in secondary metabolites, such as phlorotannins, sterols and fatty acids, all of them associated with important biological activities (Seca et al., 2016).

1.2.2. Brown macroalgae metabolites

Brown algae play important roles in marine ecosystems as predominant primary producers in the food chain and as underwater canopies for marine organisms (Sun et al., 2014). Since they inhabit intertidal zones, an environment of rapidly changing physical conditions due to the turning tides, they need to develop a series of metabolites that will help them endure this highly competitive and aggressive environment, through the production of quite specific and potent bioactive molecules, which may be a valuable resource as nutritional or pharmaceutical supplements (Paiva et al., 2018).

The secondary metabolites present in brown algae are lacking nitrogenous derivatives and have a very small number of halogenated compounds. It is possible to distinguish three main families of secondary metabolites: terpenes, phenolic derivatives, and meroterpenes, which combine aromatic derivatives (phenols, quinones, hydroquinones) with terpenes (Kornprobst, 2014).

Brown macroalgae are one of the richest sources of biologically and ecologically relevant terpenoids, mainly diterpenes and meroditerpenes; some of these compounds have proven to exhibit antifouling effects against a variety of colonizing organisms (Gaysinski et al., 2015).

One of the main features of brown algae is the presence of many phenolic derivatives, (Kornprobst, 2014). These compounds are metabolites characterized as molecules containing hydroxylated aromatic rings and show a wide variety of chemical structures. They have gained attention due to their pharmacological activity and health-promoting benefits since they play a significant role in a variety of macroalgal biological activities (Cotas et al., 2020).

Polyphenolic compounds have become common constituents of human diet due to their therapeutic properties (antioxidative, antibacterial, anti-inflammatory, among others). Several studies have confirmed the attractive antioxidant features in fresh algae (Machu et al., 2015), Puspita et al. (2017) found promising potential antioxidant activities while

analysing crude extracts of *Sargassum* algae and Miranda et al. (2018) observed antioxidant and anti-cancer activities of extracts from brown algae from Chilean coasts. When algal products are applied to food goods, Machu et al. (2015), also observed that the phenolic content of commercial products from brown algae was relevant from the nutritional point of view. However more studies on the application of polyphenolic compounds extracted from brown algae as functional ingredients, as well as the analysis of their antioxidant capacity are needed.

1.2.2.1. Phlorotannins

Tannins are a wide spread group of phenolic metabolites that contain a large number of hydroxyl groups; phlorotannins are polymeric derivatives of phloroglucinol, which are found only in brown algae; they are highly soluble in water and bind strongly to proteins, polysaccharides, and other biopolymers, they also chelate divalent metals, and have a polymeric structure (Imbs & Zvyagintseva, 2018).

The phlorotannins, mostly present in the orders Fucales and Laminariales, are polyphenols later derived from phloroglucinol and are included in organelles known as physodes, from which they are partially or fully excreted into the marine environment (Kornprobst, 2014). Phlorotannins are divided into five groups depending on inter-phloroglucinol linkage: fucols (phenyl linkage), phlorethols (ether linkage), fuhalols (ether linkage; they contain one or more additional hydroxyl group), fucophloroethols (ether and phenyl linkage) and eckols (dibenzoidin linkage), the chemical structures of selected phlorotannins can be seen in Figure 1.3.

The content of phlorotannins in brown algae reaches 15% of the dry weight, depending on the habitat of the algae, the time of their sampling, the intensity of illumination, and other factors (Imbs & Zvyagintseva, 2018).

Phlorotannins have potent antioxidant and anti-inflammatory activity (Kornprobst, 2014); and exert a physiological effect on herbivores, inhibiting digestive enzymes and protecting algae from infections; it is also suggested that phlorotannins contribute to the protection of plants from ultraviolet radiation, since they absorb a portion of the UV-B spectrum (Imbs & Zvyagintseva, 2018).

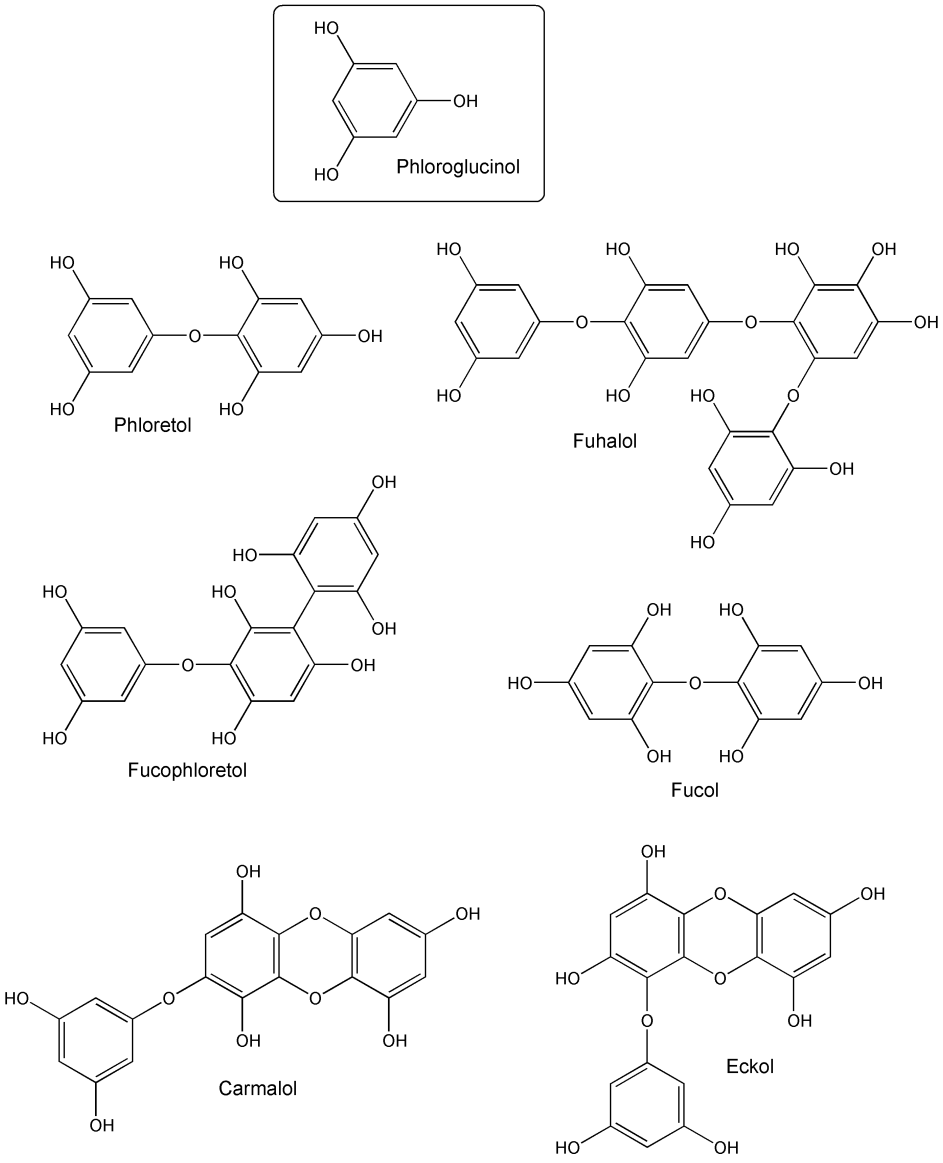

Figure 1.3: Examples of phlorotannins present in brown algae.

Although the use of phlorotannins' extracts in the formulation of functional foods is not extended due to the difficulty in their production at an industrial scale, they have shown to be strong bioactive agents that have health promoting qualities when applied to food products. Brown algae powder has been applied to pork meat, beef, chicken products and fish in oil-enriched mayonnaise (Honold et al., 2016) and granola bars (Hermund, 2016), as well as other bakery products (Roohinejad et al., 2017), and have given relatively positive results due to the antioxidant properties of the phlorotannins present in the algal powder. The use of phlorotannin enriched extracts as active food package material has also been developed to effectively preserve the quality of food products and extend their shelf-life

without the need of previously used artificial antioxidants (Cassani et al., 2020). Nanofibers with encapsulated phlorotannins have been used to protect chicken from *Salmonella* contamination as well as to improve their organoleptic quality when compared to samples protected by synthetic preservatives (Surendhiran et al., 2019). White shrimps immersed in a 5% phlorotannin solution showed a higher acceptability by consumers and an inhibitory effect against polyphenol oxidase activity and melanosis formation during ice storage, extending the shelf-life of the treated samples for four more days (Sharifian et al., 2019).

1.3. Extraction of bioactives from macroalgae

For the extraction of the compounds of interest from macroalgae, conventional methods can be used, as well as alternative methods, often classified as green methods due to the benefits they present over conventional methods, by reducing the time needed for extraction and the amount of solvent used as well as avoiding the use of non-ecological solvents (Ciko et al., 2018).

1.3.1 Conventional extraction methods

Some conventional extraction methods include techniques of hydro-distillation using water and/or steam, Soxhlet extraction, maceration, percolation, among others. However, these methods are highly dependent on the type of solvent used, sample size or the solvent/sample ratio, as well as needing longer extraction periods and presenting low extraction yields and decomposition of thermolabile ingredients.

Generally, solvent extraction is the most common method used for the extraction of compounds of interest. It requires large amounts of solvent and is dependent on the solubility of the compounds. Phlorotannins are water soluble, so highly polar solvents can be used, like water, alcohol and acetone. Lipophilic compounds like vitamins and carotenoids need low-polar solvents, such as hexane. When the extraction is carried out for subsequent use in food, pharmaceutical or cosmetic industry, ethanol aqueous mixtures are preferred (Mekinić et al., 2019).

1.3.2 Alternative extraction methods

To overcome the setbacks of conventional extraction methods, alternative techniques, including Supercritical-Fluid Extraction, Microwave-Assisted Extraction, Pressurized-Liquid Extraction, Enzyme-Assisted Extraction, and Ultrasound-Assisted Extraction have been studied (Sosa-Hernández et al., 2018).

Supercritical-Fluid Extraction (SFE) uses solvents at temperatures and pressures above their critical points, it is fast, efficient and allows the selective extraction of natural compounds from several matrices while not requiring the use of environmentally damaging organic solvents, using carbon dioxide and supercritical CO_2 instead (Jumaah et al., 2015).

Microwave-Assisted Extraction (MAE) combines conventional solvent extraction with microwave heating (Sarfarazi et al., 2020), where microwave radiation is applied at a frequency near 2.45 GHz (12 cm), causing dielectric heating and resulting in temperature increases in the intracellular liquids, evaporating the water and exerting pressure on the cell walls causing cell disruption (Kapoore et al., 2018), leading to an efficient and fast extraction of natural products.

Pressurized-Liquid Extraction (PLE) uses liquid solvents at elevated temperatures and pressures, improving the solvation of the target compound and increasing diffusion rates, minimizing the extraction time and solvent consumption. It is mainly used for the extraction of polar compounds and generally uses water and ethanol as solvents (Okiyama et al., 2018).

Enzyme-Assisted Extraction (EAE) depends on the characteristic property of enzymes to carry out reactions with accurate specificity while retaining their biological potency. Its principle is the disruption of plant cell walls by hydrolysing them using enzymes as catalysts in order to release intercellular components. This technique reduces the requirement of hazardous solvents and the extraction time (Nadar et al., 2018).

Ultrasound-Assisted Extraction (UAE) uses acoustic waves in the kilohertz range that travel through the solvent producing cavitation bubbles that burst at the surface of the plant sample matrix, inducing shockwave damage to plant cell wall and enhancing the mass transfer of phenolic compounds across cellular membranes into the solution (Al Jitan et al., 2018).

1.3.3 Extraction of Phlorotannins

Phlorotannins are usually extracted from fresh macroalgae or lyophilized material using ethanol or acetone, or their aqueous solutions. Subsequently, there is a separation of the extract by liquid extraction with organic solvents, often ethyl acetate, obtaining an extract rich in phlorotannins (Imbs & Zvyagintseva, 2018).

Microwave-Assisted Extraction (MAE) has been used to selectively extract phlorotannins from *Fucus vesiculosus* using hydroethanolic solvents, allowing a similar recovery of phlorotannins from the macroalgae compared to conventional extraction methods, obtaining extracts with identical phlorotannins constituents and with moderate scavenging abilities (Amarante et al., 2020).

Enzyme-Assisted Extraction (EAE) was used to extract these phenolic compounds from *Sargassum* spp., while comparing this method to the widely used conventional Solid-liquid extraction; revealing that by using enzymes, the efficiency of the extraction increased, improving the quality and quantity of extracts, also increasing their bioactivity (Puspita et al., 2017).

Ultrasound-Assisted Extraction (UAE) was employed to extract phlorotannins and polysaccharides from *Silvetia compressa*, significantly enhancing their extraction using only hydroethanolic solvents (Vázquez-Rodríguez et al., 2020).

Other alternative methods have been tested recently to improve the amount of phlorotannins extracted from algae material, such as subcritical water hydrolysis, centrifugal partition extraction (CPE), supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE), but the high cost of these methods and low availability reduces the possibility to apply them (Gall et al., 2015).

1.4. Natural antioxidants for the prevention of fish products oxidation

The quality of commercialized fish products has improved remarkably by increasing their oxidative stability via the development of effective control methods against lipid oxidation, including the inhibition of free radical autoxidation by the addition of antioxidants, which can be synthetic or natural. Although synthetic antioxidants are efficient and relatively cheap, there are safety concerns and negative perceptions that have brought attention to natural sources of antioxidants, like tocopherols and extracts of a wide variety of terrestrial

plants, but their use is limited due to their strong characteristic flavour (Miyashita et al., 2018).

Consequently, natural antioxidants derived from macroalgae have great potential for improving oxidative stability of food products, as well as improving the products' quality with other health improvement functionalities (Hermund, 2016).

1.4.1 Lipid oxidation in fish

Lipid oxidation in food products, such as fish, is a process where the polyunsaturated fatty acids present in the products react with ROS, otherwise known as reactive oxygen species, and lead to the degradation of lipids and development of oxidative rancidity (Amaral et al., 2018).

It occurs through pathways mediated by free radicals and enzymes or without them. It is initiated when a fatty acid or a fatty acyl side chain of any chemical entity is attacked by free radicals, that will abstract a hydrogen atom from a methylene carbon belonging to these fatty side chains, initiating the oxidation reaction (Khanum & Thevanayagam, 2017).

PUFA's or polyunsaturated fatty acids contain two or more double bonds and are very susceptible to oxidation by reactive agents; and when an allylic hydrogen, belonging to the carbon adjacent to the double bond, is removed by a reactive species, it leads to the formation of lipid peroxyl radicals. These radicals can react with a second PUFA and lead to the spread of lipid oxidation (Korkmaz, 2018). This propagation mechanism can occur up to 100 times before two radicals combine and terminate the process (Figure 1.4) (Amaral et al., 2018).

The process of oxidation produces many by-products, such as primary products, like hyperoperoxides, which are unstable and susceptible to decomposition. Final products consist of a number of complex mixtures, like aldehydes, alcohols, organic acids and epoxy compounds. Other products of lipid oxidation, like malonaldehydes and cholesterol oxides have toxic properties (Khanum & Thevanayagam, 2017), such as potentially genotoxic activities (generating base-pair mutations and DNA-protein cross-links in mammalian cells) that may lead to mutations and subsequently to cancer (Del Rio et al., 2005).

All of these products make the process of lipid oxidation one of the most important alterations that occur during processing and conservation of dietary products, since the

emergence of characteristic odours and flavours of rancidity diminishes the acceptability of food, also the ingestion of non-volatile compounds generated can have negative effects over nutritional quality and consumers health (García, 2018).

Fish contains high quantities of unsaturated lipids, which can decrease the risk of mortality from coronary heart disease and sudden cardiac death in consumers, but increase the susceptibility to degradation and oxidation of the products (Secci & Parisi, 2016).

Since fish products are considered a significant source of nutrients, and their content of long-chain omega-3 polyunsaturated fatty acids has demonstrated to contain protective effects against cardiovascular diseases, the necessity to find techniques to diminish the deterioration caused by the products from lipid oxidation arises, in order to avoid the undesirable sensory and biological effects, as well as rancidity caused by the toxic compounds generated by the oxidation of the PUFA's present in fish (Xu et al., 2015).

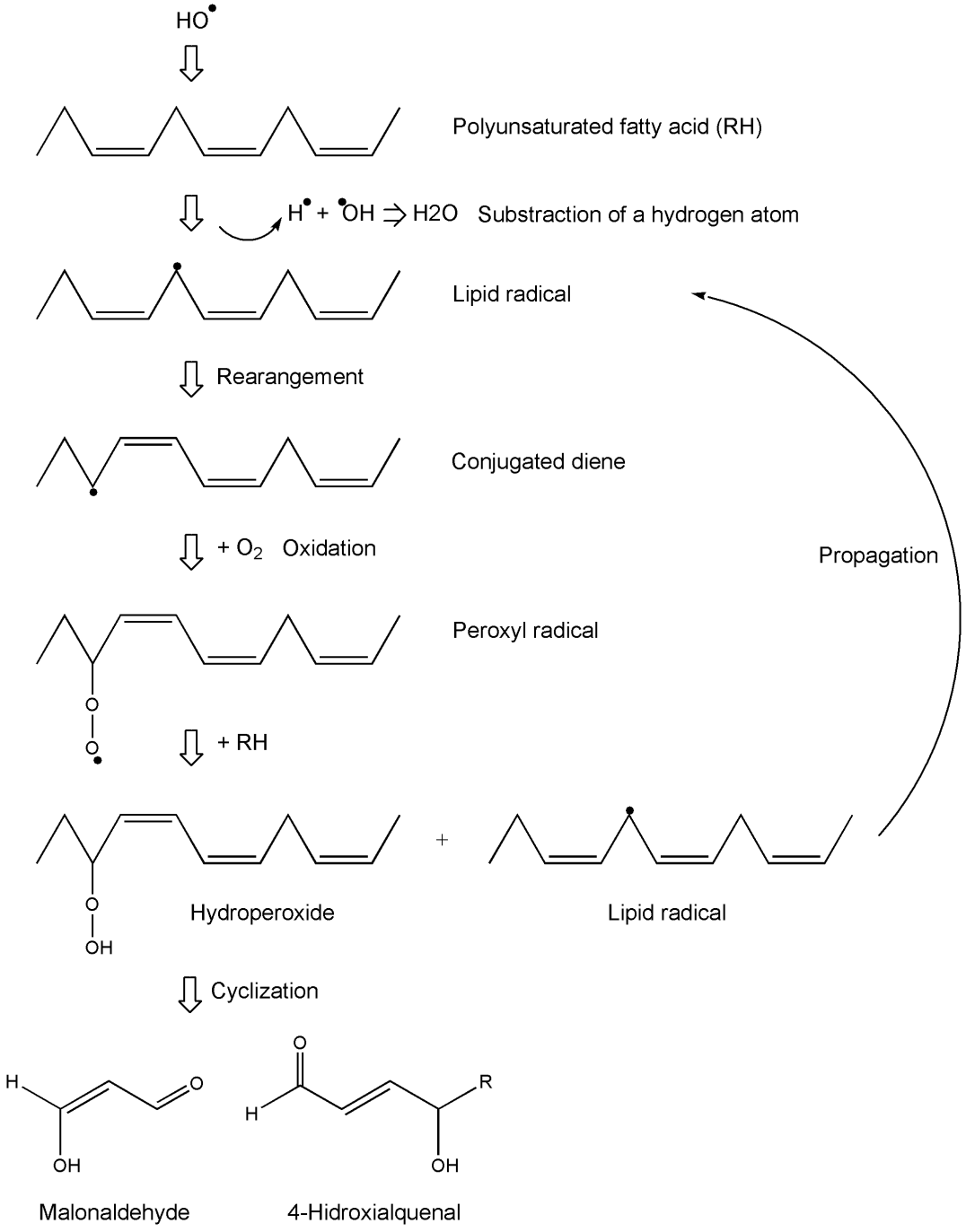

Figure 1.4: Lipoperoxidation of polyunsaturated fatty acids.

Adapted from López et al., (2011)

1.4.2 Algae-derived antioxidants for the prevention of lipid oxidation in fish products

As previously refereed, brown algae belonging to the *Fucus* genus, including *F. vesiculosus* (Karadağ et al., 2017) and *F. spiralis* (Miranda et al., 2016), are rich in bioactive polyphenolic secondary metabolites, such as phlorotannins, and other substances like pigments, for example carotenoids, tocopherols, sulphated polysaccharides, peptides, and polyphenols, all of which exhibit antioxidant activity (Karadağ et al., 2017).

These antioxidants present in brown algae, inhibit lipid oxidation in food products by transforming free radicals into non-radicals by donating electrons and hydrogen molecules or by chelating transition metals (Farvin & Jacobsen, 2015).

Phlorotannins have great antioxidant activity due to their chemical structure, they consist of up to eight interconnected rings, or phloroglucinol units, that are effective in preventing oxidation due to the higher number of functional -OH groups (Honold et al., 2016).

There have been a few previous studies using extracts of brown macroalgae, to diminish the oxidation effects in several food products, as can be seen on Table 1.1. However, there is still not enough information on the use and application of extracts obtained from *F. spiralis*' extracts on foods (Honold et al., 2016).

Algae	Title	Reference
F. vesiculosus Linnaeus	Inhibition of Hemoglobin-Mediated Lipid Oxidation in Washed Cod Muscle and Cod Protein Isolates by *Fucus vesiculosus* Extract and Fractions	Wang et al. (2010)
	Potential seaweed-based food ingredients to inhibit lipid oxidation in fish-oil-enriched mayonnaise	Honold et al. (2016)
	Characterization and antioxidant evaluation of Icelandic *F. vesiculosus* extracts in vitro and in fish-oil-enriched milk and mayonnaise	Hermund et al. (2015)
	The effect of novel brown seaweed-based antioxidants on the oxidative stability and microstructure of 5% fish-oil-enriched granola bars	Karadağ et al. (2017)
	High quality fish protein hydrolysates prepared from by-product material with *Fucus vesiculosus* extract	Halldorsdottir et al. (2014)
F. vesiculosus Linnaeus	Effect of tuna skin gelatin-based coating enriched with seaweed extracts on the quality of tuna fillets during storage at 4 °C	Vala et al. (2017)
Cystoseira compressa	Effectiveness of a combined ethanol-aqueous extract of alga *Cystoseira compressa* for 4 the quality enhancement of a chilled fatty fish species	Oucif et al. (2018)
Laminaria sp.	Brown seaweed (AquaArom) supplementation increases food intake and improves growth, antioxidant status and resistance to temperature stress in Atlantic salmon, *Salmo salar*	Kamunde et al. (2019)
Sargassum kjellmanianum	Prevention of fish oil rancidity by phlorotannins from *Sargassum kjellmanianum*	Yan et al. (1996)
Padina boergessenii	Antibiotic resistant *Escherichia coli* strains from seafood and its susceptibility to seaweed extracts	Kumaran et al. (2010)

Table 1.1: Review on the use of brown algae extracts to inhibit degradation on fish products.

2. Aims and experimental design

This thesis is divided in two parts:

i) Extraction and characterization of phlorotannins-enriched extracts from brown macroalgae *F. spiralis* collected in the four seasons of the year.

ii) Case study: effects of the incorporation of phlorotannins-enriched extracts in homogenised preparations of fresh salmon during storage.

The main aims are:

1) Use an efficient and green extraction method for the obtention of phlorotannins enriched extracts from the brown macroalgae *F. spiralis*, using food-compatible solvents;
2) Perform chemical and functional characterization of the extracts obtained to validate the correlation between phenolic compound content and antioxidant activity;
3) Analyse seasonal variations in the content of phenolic compounds and antioxidant activity of the extracts;
4) Analyse the antioxidant capacity of the phlorotannins enriched extracts when used as natural antioxidants in the prevention of salmon patties degradation.

The different phases of the work described in this thesis are schematized in Figure 2.1.

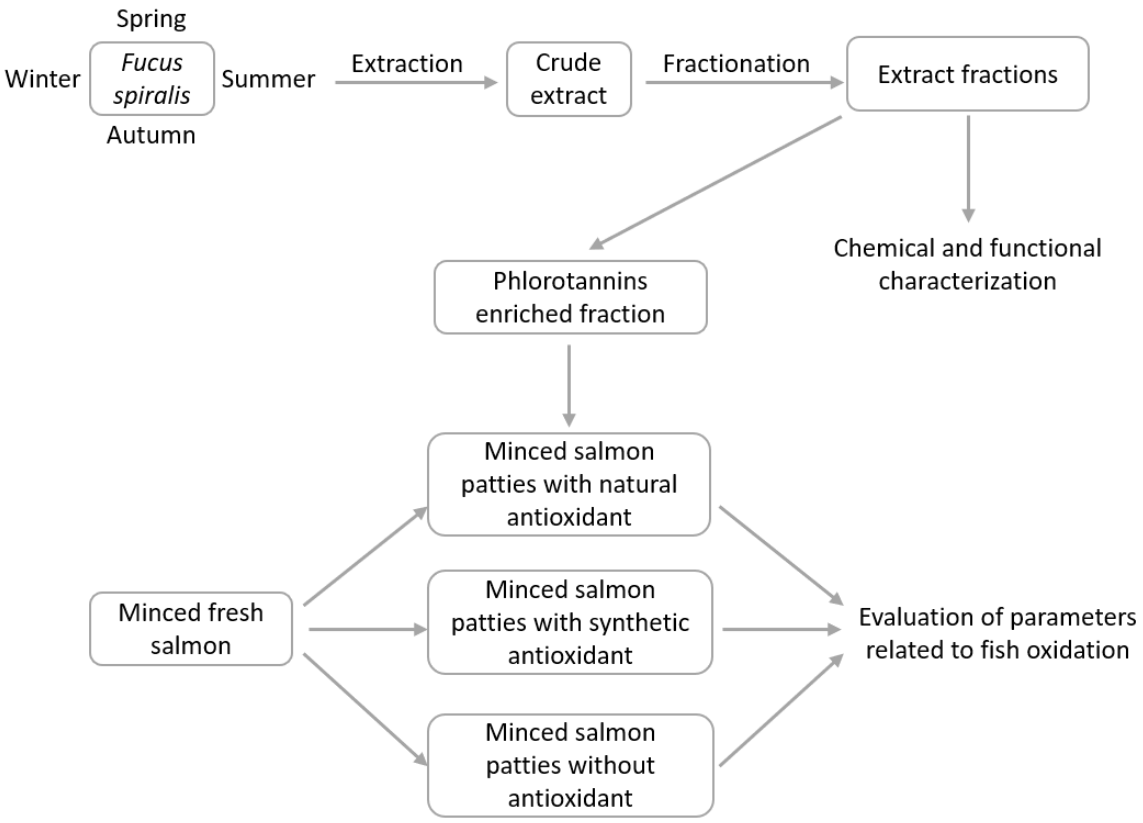

Figure 2.1: Phases carried out during the study, including extraction of crude ethanolic extract and its fractionation, and the application of an enriched extract to a food product.

3. Materials and methods

3.1 Biomass and reagents

Brown macrolagae *F. spiralis* was collected in the four seasons (autumn, winter, spring and summer) during the years of 2018/2019, in the upper intertidal zone of the Marques Neves beach (39°22'13.5"N 9°23'14.8"W), Peniche (Portugal), during low tide. The algae were washed with salt water (35% (w/v)) to remove invertebrates and other organisms, sand, and debris, frozen at -80 ºC and freeze-dried. The freeze-dried algae were finely crushed with a laboratory blender (Bimby vorwerk, thermomix 31-1) and stored in a dry recipient in the absence of light until further studies.

Fresh salmon (*Salmo salar*) was purchased from a local market.

Extra-pure grade solvents used for the extractions were purchased from commercial suppliers. Folin-Ciocalteu reagent, Gallic acid, DPPH (2,2-difenil-1-picrilhidrazil), TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine), Phloroglucinol, Pyrocathecol, Ascorbic acid, and Glucose were obtained from Sigma-Aldrich.

3.2 Extraction and semi-purification

The extraction procedure described below were performed for *F. spiralis* biomass obtained in the four seasons.

3.2.1 Ethanolic extraction

Firstly, 30 g of biomass were suspended in 240 mL of an EtOH/H_2O mixture (80:20 v/v) (biomass:solvent ratio 1:10 w/v). The suspension was mixed thoroughly for 30 min with the help of a magnetic stirrer, at room temperature. After extraction, the suspension was filtered (filter paper VWR 2-3 µm) with the help of a vacuum pump. The solvent was removed by reduced pressure using a rotary evaporator. The dried extract (Et80) was weighted and stored until further tests.

3.2.2 Liquid-liquid semi-purification of the extract Et80

Crude ethanol-water (80:20) extracts (Et80) of *F. spiralis* were fractionated using a liquid-liquid separation procedure based on polarity adapted from Gall et al. (2015), in order to obtain phlorotannin-enriched fractions. In a centrifuge tube, 1.5 g of the Et80 extract were dissolved in 40 mL of distilled water. Then, 40 mL of n-hexane were added and the mixture was shaken in a separating funnel, obtaining two phases, an aqueous phase (AQ1) and an n-hexane phase (He). The aqueous phase AQ1 was extracted using the same amount of n-hexane two more times. The n-hexane phases (He) were combined and the solvent was removed by reduced pressure using a rotary evaporator.

The aqueous extract AQ1 was reduced to about 50 mL using a rotary evaporator. A sample of 10 mL of AQ1 were taken, the solvent was removed under reduced pressure and the residue was stored for analysis. To the remaining 40 mL of AQ1, 80 mL of ethanol were added and the mixture was thoroughly mixed for 15 min and stored at -20 °C for 24 h. The mixture was then centrifuged at 8000 rpm and 4-5 °C for 20 min. The pellet was discarded, and the process was repeated until no pellet was obtained after centrifugation. Ethanol was completely evaporated to give the second aqueous phase (AQ2). 10 mL of AQ2 were evaporated and the residue kept for further analysis.

Around 120 mL of acetone were added to 40 mL of the aqueous phase AQ2, the mixture was thoroughly mixed for 15 min and stored at -20 °C for 24 h. The mixture was centrifuged at 8000 rpm and 4-5 °C for 20 min. The pellet was again discarded, and the process was repeated until no pellet was obtained after centrifugation. Once there was no pellet, the acetone was removed using a rotary evaporator, and the third aqueous phase (AQ3) was obtained. 10 mL of aqueous phase AQ3 were taken, the solvent was evaporated, and the residue stored for further analysis. 40 mL of ethyl acetate were added to the aqueous phase AQ3 resulting in two phases, an aqueous phase (AQ4) and an ethyl acetate phase (EA). The aqueous phase was extracted two more times using the same volume of ethyl acetate. The EA phases were combined and the EA and AQ4 phases were evaporated to dryness with the help of a rotary evaporator.

3.3 Characterization of extracts

3.3.1 Total Phenolic Content (TPC)

For the evaluation of the phenolic content of each extract, the Folin-Ciocalteu method was used. The Folin-Ciocalteu reagent reacts in the presence of certain reducing agents, such as phenolic compounds, in an alkaline medium, forming a blue complex. The intensity of the colour is correlated with the amount of reducing agents, and can be measured via absorbance (Margraf et al., 2015).

The content of phenols in the sample was determined using the method described by Li et al. (2017) with some modifications. A standard curve using gallic acid was created, using solutions with the following concentrations: 1, 0.3, 0.1, 0.03, and 0.01 mg/mL.

Samples of the extracts with a concentration of 5 mg/mL (EtOH/H_2O, 80:20 v/v) were used.

The determination of TPC was done using a 96-wells plate, putting 2 µL of sample, 158 µL of distilled water and 10 µL of Folin reactive in each well. After complete dissolution, it was left to incubate during 2 min at room temperature. 30 µL of Na_2CO_3 20% were added to each well and the mixture was left to incubate for one hour at room temperature. Four replicates were conducted for each extract/standard concentration. The absorbance was read at 755 nm. Blanks were made by replacing the Folin reactive for distilled water.

The TPC is expressed as mg equivalents of gallic acid per gram of extract (GAE/g extract).

3.3.2 Reversed phase high performance liquid chromatography (RP-HPLC)

The extracts were analysed by reversed phase HPLC (RP-HPLC), using a Merck-Hitachi Elite LaChrom HPLC system equipped with a L-2450 DAD detector, a L-2200 autosampler and a L-2130 pump. The analyses were carried out at room temperature with an ACE Advanced Chromatography Technologies Ltd HPLC C18 column (250 x 4.6 mm diameter size, 5 µm particle size, 100 Å pore size) equipped with a matching guard cartridge. The volume of injection was 70 µL. A gradient mobile phase consisting of formic acid in water (A, 1% v/v) and acetonitrile (B) was used, at a flow rate of 1 mL/min. The composition for a 90 min. run was as follows (Olate et al., 2019): 0-5 min. 5% B; 5-60 min.,

5- 30% B (linear); 60-70 min B, 30-60% B (linear); 70-80 min, 60% B; 80-90 min, 60-5% B (linear). The detection of aromatic compounds was made at 254 and 280 nm.

3.3.3 Proton nuclear magnetic resonance (^{1}H NMR)

^{1}H NMR spectra of the ethyl acetate extracts were measured on a Bruker Avance III 400 MHz instrument equipped with a 5-mm broad-band diffusion probe (DifBB), using D_2O as solvent, at a temperature of 298 K. The 1D ^{1}H experiments were performed using the standard Bruker parameters for zgesp for water suppression and the chemical shifts were expressed in ppm, using the residual solvent peak as reference (in the standard proton experiment).

3.4 Antioxidant capacity

3.4.1 Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay is a direct method for measuring the combined antioxidant activity of reductive antioxidant in a sample; this assay uses the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) as the indicator since it is tied to a colour change (Benzie & Devaki, 2017).

The method used was described by Puspita et al. (2017), with some modifications. A standard curve using ascorbic acid was created using solutions with the following concentrations: 1000, 750, 500, 200 and 20 µM.

Samples of the extracts dissolved in EtOH/H_2O (80:20 v/v) with concentrations of 1 mg/mL (Et80, AQ1, AQ2, and AQ3), 5 mg/mL (AQ4 and He) and 0,5 mg/mL (EA) were used.

The following solutions were prepared: A 300 mM acetate buffer (A) (pH 3.6), TPTZ 10 mM (B), and a solution of $FeCl_3$:$6H_2O$ (C). For the reagent TPTZ, the solutions A, B and C were mixed in a ratio of 10:1:1, and the mixture was kept away from light.

The assay was done in a 96-wells plate, mixing 5 µL of sample and 195 µL of TPTZ reagent in each well. Four replicates were conducted for each extract/standard concentration. The plate was left to incubate at room temperature for 4 hours and the

absorbance was read at 593 nm. Blanks were made replacing the TPTZ reagent with distilled water.

The FRAP is expressed as µM equivalents of ascorbic acid per gram of extract (µM AAE/g extract).

3.4.2 DPPH (2,2-diphenyl-β-picrylhydrazyl) radical scavenging ability

This method analyses the antioxidant activity based on the measurement of the scavenging ability of antioxidants toward 2,2-diphenyl-β-picrylhydrazyl. The odd electron of a nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine, giving a deep violet colour. When mixing DPPH with another substance that can donate a hydrogen atom, such as a phenol, there is a loss of the violet colour (Kedare & Singh, 2011).

The DPPH radical scavenging ability was analysed with the method according to Cruces et al. (2016) with some modifications. DPPH solution was made dissolving 1,6 mg of DPPH in 40 mL of methanol.

Samples of the extracts with a concentration of 5 mg/mL (EtOH/H_2O, 80:20 v/v) were used.

The assay was conducted using 96-wells plates, with a control that consisted of eight replicates of 2 µL of ethanol and 198 µL of DPPH solution. 2 µL of the sample and 198 µL of DPPH solution were mixed in each well, ensuring the homogenization of the solution. Four replicates were done for each extract. The plate was left to incubate at room temperature for 30 minutes protecting it from light and after that period the absorbance was read at 517 nm. Blanks were made replacing the DPPH solution with distilled water.

The DPPH radical scavenging ability is expressed as reduction % of the DPPH radical.

Phloroglucinol, Pyrocathecol, Gallic acid, Ascorbic acid, and Glucose were used as reference standards (1 mg/mL).

The antioxidant activity of the extracts was defined as the capacity to eliminate free radicals, and determined using equation 1:

$$Scavenging\ ability\ (\%) = \left[\frac{1-\left(Abs_{sample}-Abs_{sample\ blank}\right)}{Abs_{control}}\right] \times 100 \qquad \text{(Eq. 1)}$$

IC_{50} was determined for the extracts presenting higher capacity to eliminate free radicals, Et80, EA and AQ3, using ascorbic acid as a comparative standard.

The IC_{50} value is calculated as the concentration of the extract required to attain scavenging of 50% of the DPPH in the reaction mixture (Li et al., 2017). The IC_{50} curve was obtained by determination of the DPPH radical scavenging ability of the selected extracts ($EtOH/H_2O$, 80:20 v/v) with the following concentrations: 0,1; 0,3; 0,5; 1; 3; 5; 10; and 30 mg/mL.

3.5 Preparation of minced fresh salmon patties with incorporation of phlorotannin-enriched extracts

Atlantic salmon (*Salmo salar*) fillets were properly cleaned of bones and skin and grounded with the help of a food processor (bimby vorwerk, thermomix 31-1) until it reached desired homogeneity. The minced salmon was divided into three equally distributed groups: G1 with the incorporation of 0.01% *F. spiralis* EA extract; G2 with the incorporation of 0.01% synthetic antioxidant BHT; G3 as control group. EA extract used was from the *F. spiralis* biomass from summer season. The EA extract and BHT were dissolved in $EtOH/H_2O$ (80:20 v/v) and the same amount of the solvent mixture was added to the control group. For each treatment, 18 patties of 65 g were shaped, wrapped with polyethylene film paper, and stored at 4±1 °C in Petri dishes. Analysis were done in triplicate and performed at days 0, 3, 7, 11, 15, and 21 of storage, using 3 patties per group.

3.6 Physicochemical analyses

3.6.1 pH value

The pH of the salmon patties was measured directly using a previously calibrated potentiometer for solids (WTW, Inolab), by dipping the tip of the electrode of the perforation probe into each patty.

3.6.2 Moisture content

The moisture content of each patty was measured according to the method described by the Portuguese Norm 282 (1991), by oven drying the sample and measuring weight loss. Porcelain crucibles were dried for 15 minutes at 105 °C, cooled to room temperature in a desiccator and weighted. 10 g of sample were weighted into the pre-weighed crucibles. The crucibles were placed into a drying oven at 105 °C for 3 hours. The samples were then removed and allowed to cool to room temperature in a desiccator. The containers were kept inside the desiccator and weighted daily until the weight did not differ more than 0.01 g from the previous weighting. Moisture content was calculated using equation 2:

$$\%\, Moisture = \frac{m_2 - m_3}{m_2 - m_1} \times 100 \qquad \text{(Eq. 2)}$$

Where m_1 is the weight of the empty crucible, m_2 is the mass of the fresh sample and m_3 is the average weight of the dehydrated sample.

3.6.3 Colour

Colour measurement was carried out by direct reading of Cielab coordinates (L*, a* and b*) of the samples using a Konica Minolta colourimeter (CR400, Minolta, Japan) previously calibrated with the use of a white ceramic (CIE standard illuminant D65). Average colour space values were obtained by measuring three different points at the top of each patty. The value of L* represents the lightness or luminosity varying from a value of 0 (black) to 100 (white), while a* and b* are colour coordinates that vary from -60 (green) to +60 (red) for a*, and -60 (blue) to +60 (yellow) for b* respectively (Atitallah et al., 2019).

3.7 Lipid oxidation analysis

3.7.1 Total volatile base nitrogen (TVB-N)

The content of volatile basic Nitrogen was measured according to the Conway diffusion method described in the Portuguese Norm 2930:1988, where volatile bases are extracted by a solution of trichloroacetic acid and posteriorly quantified through titration with hydrochloric acid (Asensio et al., 2019).

A sample of 12.5 g of salmon patty were homogenized with 50 mL of trichloroacetic acid solution 5% for 2 minutes, followed by centrifugation at 3000 rpm at 4 ºC for 20 minutes.

An aliquot of 1 mL of boric acid containing indicator was pipetted into the inner chamber of Conway cells, and 1 mL of the sample supernatants were pipetted into the outer chambers of the cells. The cells were immediately covered. 0.5 mL of distilled water and 1 mL of potassium carbonate saturated solution were quickly added to the outer chambers and the cells were carefully rotated to mix the outer chamber solutions. The cells were incubated at 40 °C for 90 minutes. The titration of the inner chamber was carried out with hydrochloric acid 0.02 N until a pink colour was obtained. A diffusion control and a blank were conducted parallelly by following the same methodology previously described but replacing the sample with ammonium sulfate 0.1% and distilled water, respectively.

TVB-N was expressed in milligrams per 100 g of sample, and obtained by equation 3:

$$TVBN\ \left(\frac{mg}{100g}\right) = \frac{21\times(V_2-V_0)}{(V_1-V_0)\times V_3\times m}\times(100+H) \qquad \text{(Eq. 3)}$$

Where V_0 is the volume (mL) of the hydrochloric acid solution spent in the blank, V_1 is the volume (mL) of the hydrochloric acid solution spent in the diffusion control, V_2 is the volume (mL) the hydrochloric acid solution spent in the assay with the sample, V_3 is the volume (mL) of the sample supernatant used in the determination, H is the sample moisture (%) and m is the mass (g) of the sample.

3.7.2 Thiobarbituric acid reactive substances (TBARS)

The TBARS value was determined by the spectrophotometry method described in the Portuguese Norm 3356:1990, where the malonaldehyde (MA) formed by the decomposition process of the hydroperoxides in fish polyunsaturated acids is extracted with trichloroacetic acid, propyl gallate and EDTA, and quantified by the formation of a red coloured complex between MA and TBA that absorbs light at 530 nm (Cilli et al., 2020).

A sample of 15 g of salmon patty were mixed with 30 mL of solution containing 7.5% trichloroacetic acid, 0.1% EDTA, and 0.1% of propyl gallate, followed by agitation for 2 minutes in order to extract malonaldehyde. The mixture was centrifuged at 3000 rpm for 10 minutes. 0.5, 1, and 3 mL of the supernatant were pipetted to a test tube and 5 mL of the trichloroacetic acid solution were added. A blank was conducted simultaneously by replacing the sample with trichloroacetic acid solution. For the calibration curve, the sample

was replaced with a 1,1,3,3-tetraetoxipropane (TEP) solution with the following concentrations: 0.01, 0.02, 0.03, 0.04, and 0.05 µmol of MA. 5 mL of TBA 0.02 M solution were added to every test tube, followed by homogenization and incubation in a water bath at 100 °C for 40 minutes. The absorbance was read at 530 nm.

The TBARS value was expressed in mg of MA per 1000 g of sample and was obtained using equation 4:

$$TBARS\ \left(\frac{mg}{1000g}\right) = \frac{M(MA)\times n}{m\times v1} \times \left(VE + \frac{m\times H}{100}\right) \qquad \text{(Eq. 4)}$$

Where n is the quantity (µmol) of MA (obtained through the calibration curve), v1 is the volume (mL) of the sample used in every tube, m is the mass (g) of the sample used, VE is the volume (mL) of the trichloroacetic acid solution used in the extraction and H is the sample moisture (%). M(MA) equals 72.0636 g/mol.

3.8 Statistical Analysis

In order to validate the antioxidant activity of the extracts, Total Phenolic Content (TPC) and Ferric Reducing Antioxidant Power (FRAP) were obtained by the measurement of the absorbance of the samples. The concentration was calculated from linear regression analysis of the absorbance measurements, of concentration standards (namely, Gallic acid and Ascorbic acid) (Stone & Ellis, 2005).

Prior to all analysis, data were checked for normality and homoscedasticity (via the Shapiro-Wilk test and Levene test, respectively). Since they were not met, the non-parametric Kruskal-Wallis test was used followed by Dunn multiple comparison test (Zar, 2010). Whenever applicable, Dunnett's multiple comparisons test was employed to determine significant differences relatively to control treatment.

To assess the differences between extracts (Et80, He, AQ1, AQ2, AQ3, AQ4 and EA) regarding the results obtained for TPC (mg GAE/g extract) and FRAP (µM AAE/g extract) and DPPH (%) a one-way analysis of variance (Kruskal-Wallis) was performed. Also, to assess the effect of seasonality (namely, summer, autumn, winter and spring) on TPC (mg GAE/g extract), FRAP (µM AAE/g extract) and DPPH (%), a one-way analysis of variance (Kruskal-Wallis) was used (Zar, 2010). In addition, for the DPPH reductive test, the standards (namely, Gallic acid, Ascorbic acid, Phloroglucinol, Catechol and Glucose) were compared with the extracts for each season and the same procedure was performed.

Finally, to investigate the antioxidant capacity of the phlorotannins enriched extracts when used as natural antioxidants in the prevention of fresh fish degradation, also a one-way analysis of variance (Kruskal-Wallis) was performed.

The half maximal inhibitory concentration (IC_{50}) was calculated from nonlinear regression analysis using the software GraphPad InStat v5.1 (San Diego, CA, USA) with equation 5 (Finney, 1971):

$$Y = 100/\left(1 + 10^{[x - \log(IC_{50})]}\right) \qquad \text{(Eq. 5)}$$

The statistical analysis of the data was carried out using the software Rstudio AGLP v3. All the results were considered statistically significant at the 5% significance level (that is, p-value ≤ 0.05) (Dinno, 2015). Results are presented as means ± standard deviation (SD).

4. Results and discussion

4.1 Extraction and characterization

Crude ethanol-water (80:20) extracts (Et80) were first obtained from the freeze-dried brown algae *F. spiralis* of the four seasons (summer, autumn, winter and spring). The Et80 crude extracts were then fractionated using a liquid-liquid separation procedure based on polarity (Gall et al., 2015) in order to obtain the EA phlorotannin-enriched fractions (Figure 4.1). The original protocol involved an extraction step with dichloromethane that was skipped in order to achieve a greener extraction process and food-compatible solvents.

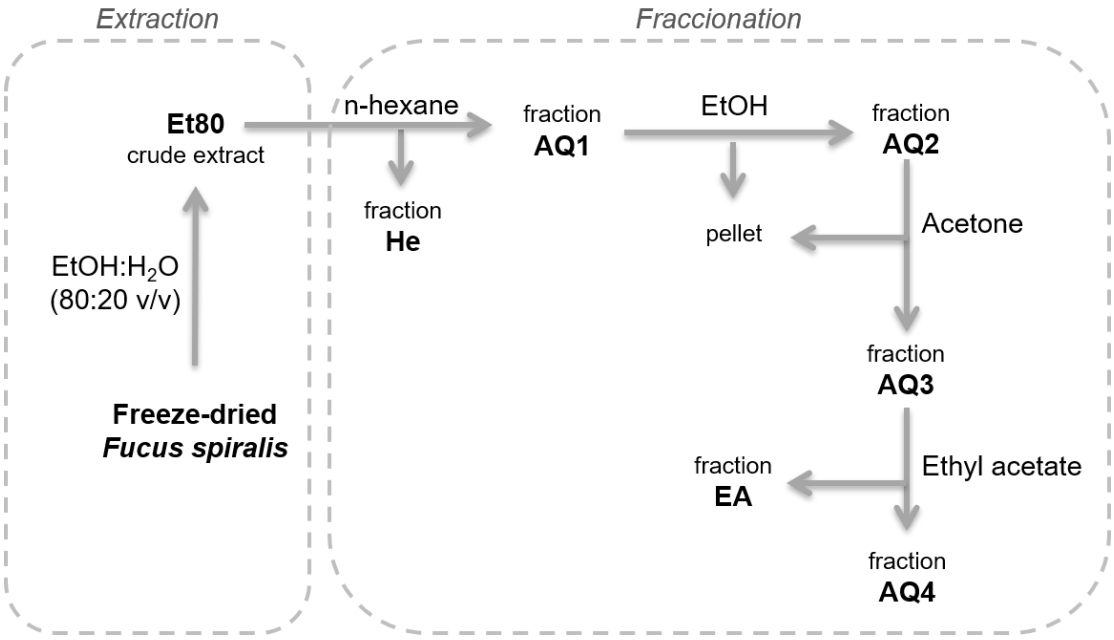

Figure 4.1: Process for the extraction and fractionation of *F. spiralis*.

All the extracts (Et80, He, AQ1, AQ2, AQ3, AQ4 and EA) obtained for *F. spiralis* collected in the four seasons were then evaluated for their antioxidant capacity and total phenolics.

4.1.1. Total Phenolic Content (TPC)

The phenolic content of the extracts obtained from *F. spiralis* was estimated using the Folin Ciocalteu test and the results are presented in Figures 4.2 and 4.3. A clear difference can be seen, not only between extracts, but also between seasons.

The extract that showed the highest amount of phenols in every season was ethyl acetate (EA), having 308.634 mg GAE/g of extract for the summer sample, 56.777 mg GAE/g of extract for the autumn season, 52.282 mg GAE/g of extract for the winter season, and 49.170 mg GAE/g of extract for the spring season.

For the samples of most of the seasons, summer, autumn and spring, the extract with the lowest phenolic content is AQ4, with 32.646, 5.602, and 2.490 mg GAE/g of extract respectively; while the Et80 extract from the winter sample has seemingly the lowest amount of phenolic content for this season, with 7.331 mg GAE/g of extract.

The results obtained for the effect of the extracts over the TPC values showed statistically significant differences (Kruskal-Wallis, χ^2=30.3444, p-value≤0.01; Fig. 4.2). The EA extract had a higher yield of TPC for every season. These differences were statistically significant for most of the comparisons between extracts (Dunn test, p-value≤0.05; Fig. 4.2).

In the summer season the effect of the extracts over the TPC value showed a statistical difference (Kruskal-Wallis, χ^2=26.2018, p-value≤0.01). The extracts AQ3 and AQ2 showed the higher TPC values after the EA extract and were significantly higher than the extract with the lower TPC yield for this season, namely, AQ4 (Dunn test, p-value≤0.05).

A statistical difference was observed in the effect of the extracts over the TPC yield for the autumn season (Kruskal-Wallis, χ^2=23.5973, p-value≤0.01). The extracts Et80, He and AQ1 had the higher TPC yield after the EA extract and were significantly higher than AQ4 which has the lower TPC yield for this yield (Dunn test, p-value≤0.05).

The statistical difference on the effect of the extracts over the TPC value for the winter season (Kruskal-Wallis, χ^2=19.4646, p-value≤0.01) showed that the extract AQ3 had the highest TPC value apart from EA and was significantly higher than the extracts with the lower yield of TPC of the season, that is Et80, He and AQ1 (Dunn test, p-value≤0.05).

The effect of the extracts over the TPC yield for the spring season also showed a statistical difference (Kruskal-Wallis, χ^2=20.7474, p-value≤0.01), where the extracts with the higher TPC yield after EA were Et80, AQ1 and AQ2 and were significantly higher than the extract with the lower TPC of the season, namely AQ4 (Dunn test, p-value≤0.05).

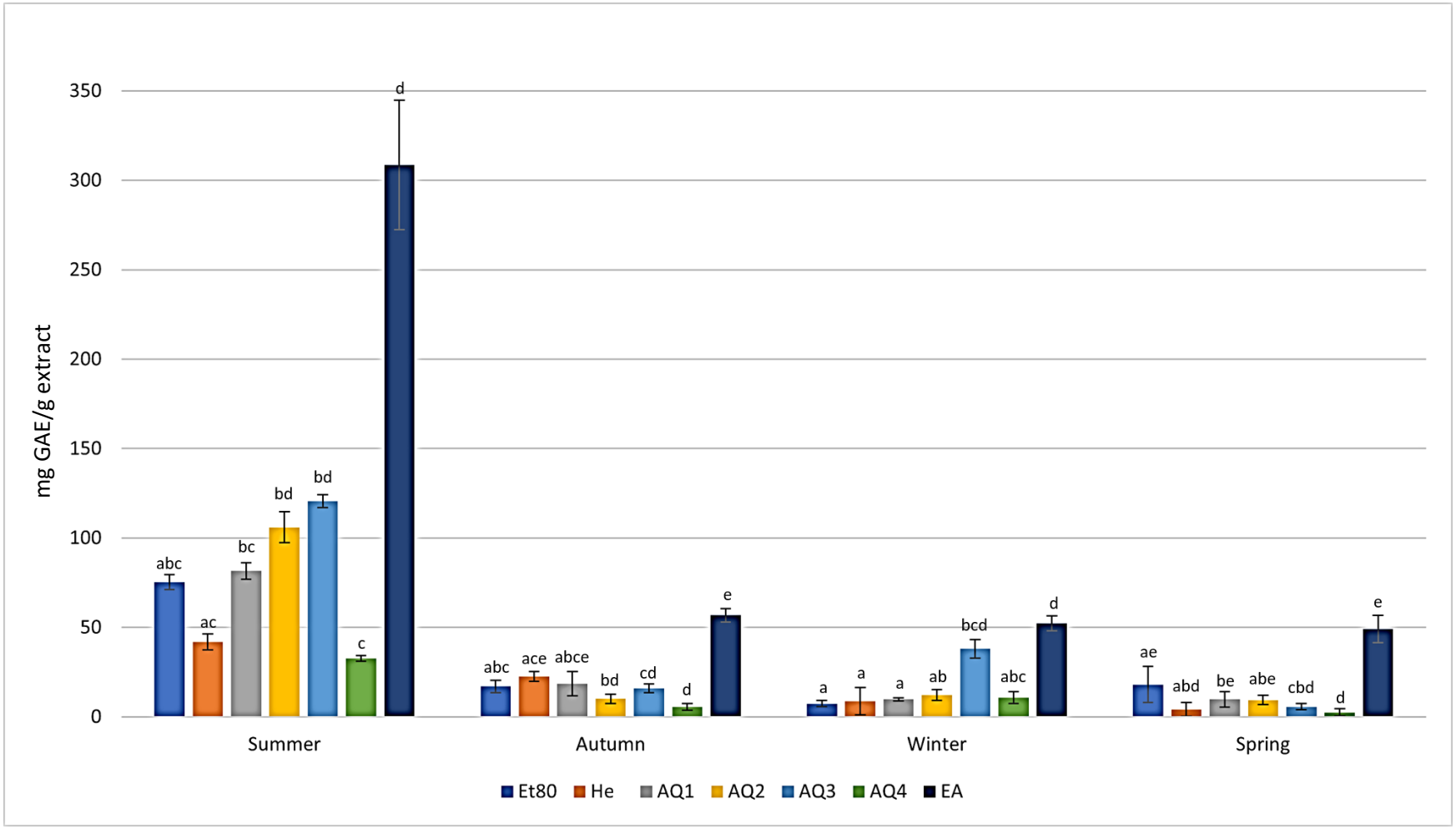

Figure 4.2: Total Phenolic Content (mg GAE/g extract) of the extracts obtained from *F. spiralis* for all the seasons. Results are represented as mean ± SD (n=4) and different lowercase letters represent significant differences (Dunn test, p-value ≤ 0.05) between extracts in each season.

Overall, the season with the highest amount of phenols in the samples is summer, with a TPC that ranges between 32.646 mg GAE/g of AQ4 extract and 308.634 mg GAE/g of EA extract. On the other hand, the season with the lowest amount of phenols seems to be spring with a TPC that ranges from 2.49 mg GAE/g of AQ4 sample and 49.170 mg GAE/g of EA sample.

The results obtained for the effect of the season on the TPC values showed statistically significant differences (Kruskal-Wallis, χ^2=54.9459, p-value≤0.01; Fig. 4.3). It was possible to observe that the summer season showed a significant difference from most of the seasons (regardless of the solvent used for the extraction of each extract) (Dunn test, p-value≤0.05; Fig. 4.3).

When analysing the effect of the seasons over the TPC yield of extracts Et80 a statistical difference could be seen (Kruskal-Wallis, χ^2=12.2345, p-value≤0.01) and it was possible to observe that the season with the highest TPC values was summer, which was significantly higher than winter, the season with the lowest TPC values of this extracts (Dunn test, p-value≤0.05).

The effect of the seasons over the TPC yield of extracts He also showed a statistical difference (Kruskal-Wallis, χ^2=11.4967, p-value≤0.01). The season with highest TPC for this extract was summer followed by autumn, both were significantly higher than the seasons with the lowest TPC yield, that is spring and winter (Dunn test, p-value≤0.05).

A statistical difference could be seen in the effect of the season over the TPC yield of AQ1 extracts (Kruskal-Wallis, χ^2=12.7434, p-value≤0.01). The summer season showed the highest TPC values for this extract and was significantly higher than the seasons with the lowest values of TPC, that is winter and spring (Dunn test, p-value≤0.05).

The statistical difference in the effect of the season over the TPC value of extracts AQ2 (Kruskal-Wallis, χ^2=9.3004, p-value≤0.01), showed that the season with the highest TPC values for this extract was summer and was significantly higher than autumn and winter, the seasons with the lowest TPC values (Dunn test, p-value≤0.05).

The effect of the season over the TPC yield of extracts AQ3 and AQ4 also showed statistical differences (Kruskal-Wallis, χ^2=14.1503, p-value≤0.01). In detail, it showed that the season with the higher TPC yield for both extracts was summer and that it was significantly higher than the seasons with the lowest TPC yields, namely spring and autumn (Dunn test, p-value≤0.05).

There was also a statistical difference in the effect of the season over the TPC value of the EA extracts (Kruskal-Wallis, χ^2=10.2434, p-value≤0.02). The summer season was the one with the higher TPC yield for this extract and was significantly higher than the seasons with the lower TPC yield, that is winter and spring (Dunn test, p-value≤0.05).

Brown algae belonging to the *Fucus* genus have shown high quantities of phenolic content in previous studies. *F. vesiculosus* aqueous extract has exhibited relatively high amounts of polyphenols, having an extra amount of 0.19 g of phloroglucinol equivalent per 100 grams of extract when compared to other brown algae, *Ascophyllum nodosum* (Agregán et al., 2017). Ethanolic extracts of *F. serratus* have also been analysed and have shown a phenolic content of 75.96±10.11 mg of gallic acid equivalent per gram of extract, significantly higher than the phenolic contents displayed by other algae studied, 1.39±0.24 mg GAE/g extract and 4.76±0.17 mg GAE/g extract present in *Laminaria digitata* and *Gracilaria gracilis* respectively (Heffernan et al., 2014). When studying fractioned extracts of *F. spiralis*, the aqueous extract showed the lowest phenolic content (8.00 ± 1.10 PGE/g extract), while the methanolic and dichloromethane extracts had the highest phenolic content (379.00 ± 34.0 and 419.00 ± 3.00 PGE/g extract) (Pinteus et al., 2017); this coincides with the results obtained in this study where the aqueous extracts, specifically AQ4 for summer, autumn, and spring, and the ethanolic extract Et80 for the winter season, have the least amount of polyphenols between all of the extracts from *F. spiralis*, while the extracts obtained with a polar aprotic solvent, in this case ethyl acetate instead of dichloromethane, exhibited a higher phenolic content that ranged between 49.170 and 308.634 mg GAE/g of extract.

This difference between the aqueous and ethyl acetate extracts may be due to the fact that algae from the *Fucus* genus are some of the brown algae with the highest amount of phenols, usually in the form of phlorotannins, even reaching up to 12% of their dry weight (Catarino et al., 2019). When these phlorotannins are the target compound of the extraction, moderately polar solvents yield better results (Pinteus et al., 2017). This was also observed by Babbar et al. (2014), where the polarity of the solvent influenced the TPC of the extracts, revealing that ethyl acetate was better than chloroform and n-hexane for the extraction of phenol enriched extracts. This was probably due to their higher polarity and better solubility for phenolic components in algae extracts. Li et al. (2017), indicated that after liquid-liquid extraction, the phenolic content in ethyl acetate extracts were higher than the content of the initial ethanolic extract while the TPC in aqueous extracts was lower, as also observed in this study.

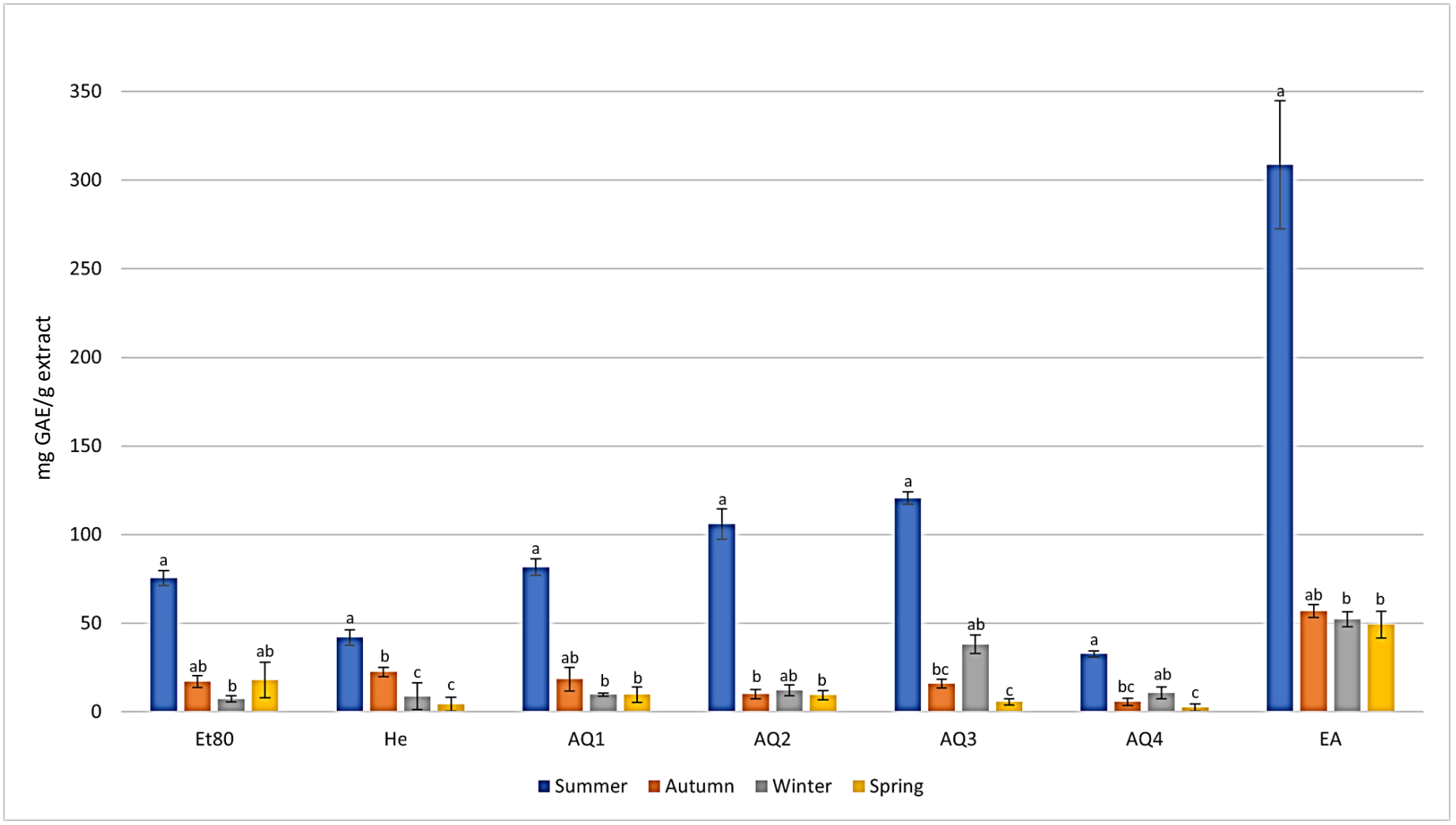

Figure 4.3: Total Phenolic Content (mg GAE/g extract) from *F. spiralis* extract, represented for each extract in the four seasons. Results are represented as mean ± SD (n=4) and different lowercase letters represent significant differences (Dunn test, p-value ≤ 0.05) between seasons for each extract.

4.1.2. Proton nuclear magnetic resonance (^{1}H NMR) analysis

The ^{1}H NMR spectra of the ethyl acetate extracts were analysed as these were the ones that presented the higher contents in phenolic compounds in all seasons (Figure 4.4). The peaks associated with aromatic compounds (such as phenolic compounds, including phlorotannins), found between 5.5 and 6.5 ppm, were more abundant in the summer EA extract, in agreement with the results obtained for the total phenolic content. Apart from the peaks associated with aromatic compounds, other peaks can be observed due to the presence of mannitol (peaks between 3.5 and 4 ppm) and other compounds such as lipids and amino acids (between 0.5 and 3 ppm).

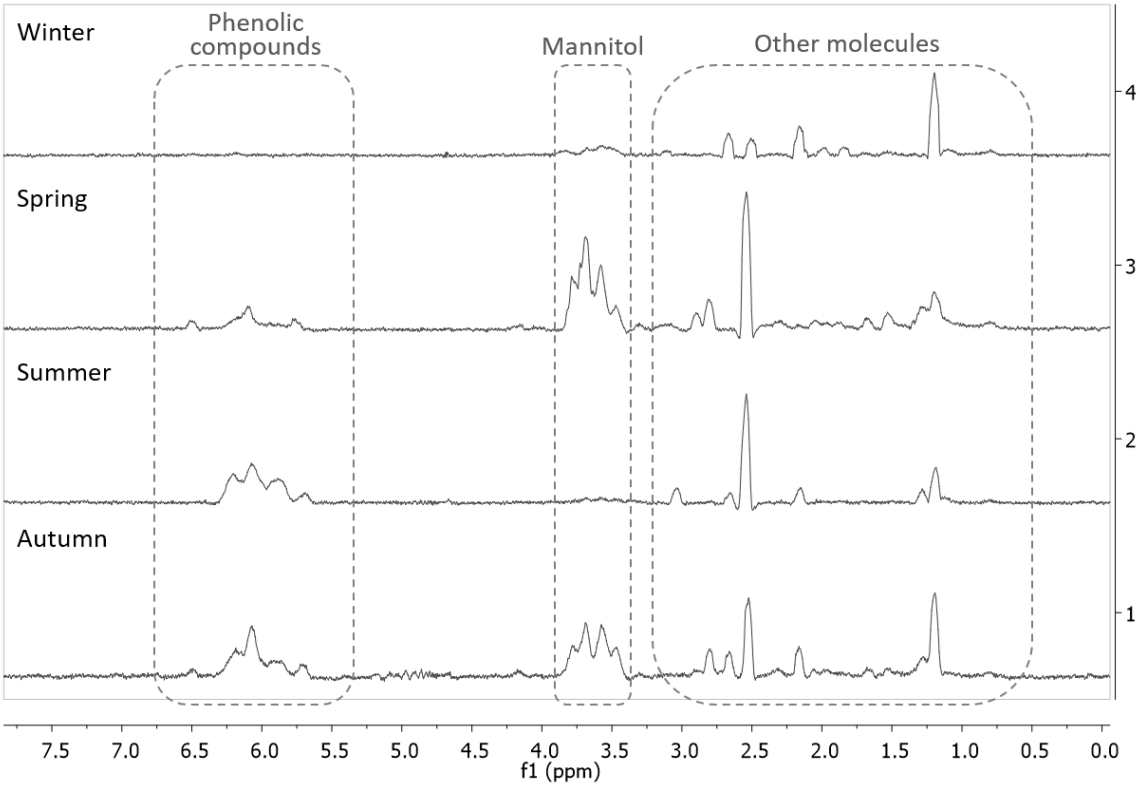

Figure 4.4: Stacked ^{1}H NMR spectra of the EA extracts of the four seasons. Spectra acquired in D_2O with water suppression.

These results are similar to the ones found by Gager et al. (2020) when analysing the ^{1}H NMR spectra of brown macroalgae, where *F. serratus* showed signals that corresponded to phenolic compounds (around 6 ppm), peaks that correspond to mannitol (4-3.5 ppm), and other peaks (below 3 ppm) that belong to other apolar molecules. Gall et al. (2015) also

found similar peaks that corresponded to the aromatic area (5.5-6.5 ppm) and to mannitol (4-3.5 ppm) in brown algae from the Fucales order. Other species of brown algae also demonstrated different peak intensity and shape depending on the season, the profiles for *A. nodosum* and *H. siliquosa* had different peaks for the autumn and winter samples, difference that can also be observed in the spectra presented by the *F. spiralis* samples of this study, due to the seasonality of the phenolic contents of these algae (Ford et al., 2019).

Jégou et al. (2015) also observed a seasonal variation of phlorotannin levels in brown algae, where the phloroglucinol content from Sargassaceae species was higher toward the autumn months. This may be due to the build-up of these phenolic compounds during the reproductive period of the algae (Stiger et al., 2004). In the present study the peak belonging to phenolic compounds is more intense in the summer season.

The quantification of phlorotannin content is not an easy task, the Folin-Ciocalteu method used for the analysis of TPC is the most common method used, however it is subject to interferences since the reagent used in the test also oxidizes several non-phenolic compounds, leading to over-estimations. This kind of method also detects the whole pool of phlorotannins present in the sample without further characterization. The NMR method can detect very specific compounds making it an innovative and rapid method to quantify phloroglucinol in brown macroalgae due to its specificity towards targeted molecules (Jégou et al., 2015). However, further purifications steps of the phlorotannin-enriched extracts would be needed in order to perform a more detailed structural characterization.

4.1.3. Reversed phase high performance liquid chromatography (RP HPLC) analysis

Algal extracts contain a large number of very similar compounds that make identification of individual structures very difficult, and although HPLC cannot be used to fully identify the structure of the phlorotannin and the position of linkages, valuable information on the distribution, type and size of phlorotannins can be obtained (Ford et al., 2019).

The application of liquid chromatography allows large-scale isolation and purification of individual compounds, however a suitable HPLC method for brown algal phlorotannins is lacking. A better separation is beneficial since it means more purified individual compounds, resulting in better isolation and identification (Koivikko et al., 2007). Once a compound of previously known beneficial activities is properly identified and isolated, it not only can be applied to food products as a functional ingredient, but it may also make the extraction of specific phlorotannins with antioxidant abilities in an industrial scale a reality.

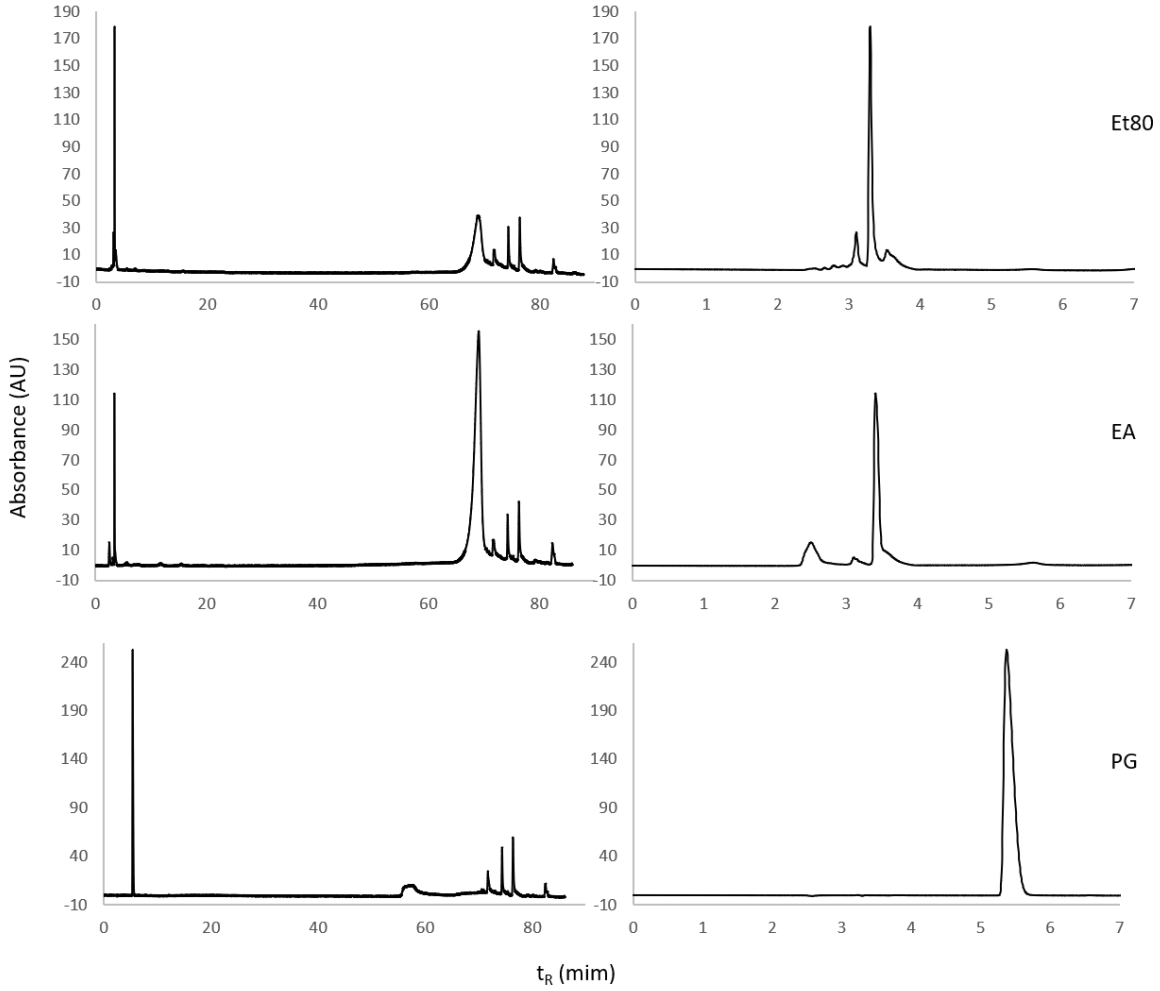

Figure 4.5: UV chromatograms of Et80 and EA extracts and of phloroglucinol (PG), using RP-HPLC with a C18 column and detection at 280 nm. The chromatograms on the right are amplifications of the chromatograms of the left, in the range t_R = 0 to 7 min.

A tentative identification of phlorotannin compounds in the Et80 and EA extracts of the summer season was carried out using RP-HPLC, following the conditions described in Olate-Gallegos et al. (2019) for the identification of polyphenols from brown macroalgae extracts (Fig. 4.5). The UV chromatograms of Et80 and EA extracts from summer season are presented in Figure 4.5. Et80 extract at 280 nm presents two peaks with retention times between 3 and 4 min, which were assigned to low molecular weight phenolic compounds (Olate-Gallegos et al., 2019). A broad peak at ca. 69 min was tentatively assigned to high molecular weight compounds, such as high molecular weight phlorotannins. The EA extract presents the same composition of the Et80 extract, but the peaks assigned to low molecular weight compounds are less intense while the peaks assigned to high molecular weight compounds appear more intense. This is consistent with EA extract being enriched in phlorotannins, comparatively to Et80 extract. A pure sample of phloroglucinol was injected

to determine the respective retention time, which was 5.4 min. The algal extracts did not exhibit the monomer phloroglucinol. The peaks beyond 70 min were attributed to solvent impurities.

The above results are consistent with what is reported in the literature. For instance Steevensz et al., (2012) reported that *F. vesiculosus* has displayed high concentrations of low molecular weight phlorotannins (< 1200 Da). Melanson & Mackinnon (2015) also found peaks of high intensity with a retention time inferior to 10 minutes, but these peaks were attributed to phlorotannins.

The difficulty of separating and identifying phlorotannins by HPLC lies in the conjunction of three factors: i) isomeric phlorotannins may present identical retention times, ii) phlorotannins of very low molecular weight are typically very polar compounds and tend to exhibit short retention times under reversed-phase conditions, while intermediate and high molecular weight phlorotannins may exhibit much longer retention times, making separation difficult (Ford et al., 2019; Heffernan et al., 2015) and iii) structurally speaking, phlorotannins are a very diverse class of compounds. While Olate-Gallegos et al. managed to separate and tentatively identify a myriad of phlorotannins extracted from different alga by reversed-phase HPLC-MS, the confirmation of the identity of a given phlorotannin compound still requires the use of pure analytical references. Due to the factors mentioned earlier, the availability analytical references of phlorotannins is scarce.

4.1.2. Antioxidant capacity

Due to the presence of different bioactive compounds with anti-oxidative potential in the extracts, different methods have been used to investigate antioxidant activity in recent years. In this work, the antioxidant activity and polyphenol content were assessed by FRAP and DPPH.

The ferric reducing antioxidant power (FRAP) assay is based on the ability of phenolic compounds to reduce Fe^{3+} to Fe^{2+}. The redox reaction occurs in the presence of 2,4,6-trypyridyl-s-triazine to induce the formation of a coloured Fe^{2+} complex, allowing the colorimetric quantification of the phenolic compounds present in the sample, in comparison with a standard of known concentration. The analysis of the antioxidant activity of the extracts using the FRAP assay can be seen in Figures 4.6 and 4.7.

The extract that gave the better results considering the FRAP test for every season was the Ethyl Acetate extract (EA), with 1723, 873.375, 360.25, and 748.375 µM AAE/g of extract for the summer, autumn, winter and spring seasons, respectively. The extract with the lowest FRAP values was the hexane extract (He) for the summer season, with 96.25 µM AAE/g of extract; AQ2 for the winter season, with 79.1875 µM AAE/g of extract; and AQ4 for the autumn and spring season, with 45.588 and 54.9 µM AAE/g of extract, respectively.

The results obtained for the effect of the extracts on the FRAP values showed statistically significant differences (Kruskal-Wallis, χ^2=31.8866, p-value≤0.01; Fig. 4.6). It was possible to observe that EA extract was significantly different from most other extracts tested from all other extracts used (regardless of the season of recollection) (Dunn test, p-value≤0.05; Fig. 4.6). In addition, it is possible to observe that the EA extract had a higher yield of FRAP for every season.

In the summer season, the effect of the extracts over the FRAP values showed a statistical difference (Kruskal-Wallis, χ^2=25.7724, p-value≤0.01). Specifically, it can be seen that the extracts with the higher yield of FRAP after the EA extract were AQ3 followed by AQ2 and that both were significantly higher than the extract with the lower FRAP yield, that is He (Dunn test, p-value≤0.05).

The effect of the extracts over the FRAP values for the autumn season also showed a statistical difference (Kruskal-Wallis, χ^2=24.8861, p-value≤0.01). The extracts with the higher value of FRAP after the EA extract were AQ3 and AQ2 and were significantly higher than the extract with the lower FRAP yield of the season, namely AQ4 (Dunn test, p-value≤0.05).

There was also a statistical difference on the effect of the extracts over the FRAP values of the winter season (Kruskal-Wallis, χ^2=24.5166, p-value≤0.01). The extract EA was the one with the higher FRAP values for this season, followed closely by the extract AQ3 and both extracts were significantly higher than the extracts with the lowest FRAP values, namely Et80, AQ2, AQ4 (Dunn test, p-value≤0.05).

In the spring season, the effect of the solvent over the FRAP values of the extracts showed a statistical difference (Kruskal-Wallis, χ^2=23.5567, p-value≤0.01); the extracts that showed the higher FRAP values after EA were AQ1 and AQ3 and were significantly higher than the extracts with the lowest FRAP values for this season, that is AQ4, Et80, He and AQ2 (Dunn test, p-value≤0.05).

Similar to the results obtained for TPC, the season with best results in FRAP assay for every extract except He was summer and the season with the lowest values was spring, since most of the extracts from this season, Et80, He, AQ2, AQ3 and AQ4, presented values of less than 100 µM AAE/g of extract.

The results obtained for the effect of the seasons on the FRAP values of the extracts showed statistically significant differences (Kruskal-Wallis, χ^2=54.1662, p-value≤0.01; Fig. 4.7). It was possible to observe that the summer season was significant different from most of the seasons (regardless of the solvent used for the extraction of each extract), except in the He extract (Dunn test, p-value≤0.05; Fig. 4.7). In addition, the results showed that the extracts from summer season had a higher yield of FRAP.

When analysing the effect of the seasons over the FRAP values of the extracts Et80 it was possible to observe statistical differences (Kruskal-Wallis, χ^2=13.8274, p-value≤0.01). The season with the highest FRAP values was summer and it was significantly higher than spring, the season with the lowest values (Dunn test, p-value≤0.05).

The season also had a statistically significant effect over the FRAP values of the He extracts (Kruskal-Wallis, χ^2=11.7305, p-value≤0.01). Unlike other seasons, summer did not show the highest FRAP values but instead, winter was significantly higher than the season with the lowest FRAP, that is spring (Dunn test, p-value≤0.05).

The effect of the season over the FRAP values for extracts AQ1 and AQ2 showed statistical differences (Kruskal-Wallis, χ^2=12.7467, p-value≤0.01). The season with higher FRAP yield for both extracts was summer and it was significantly higher than the seasons with the lowest yields, namely winter and spring (Dunn test, p-value≤0.05).

For the extracts AQ3 and AQ4 the season also showed a statistically significant effect over the FRAP values of the extracts (Kruskal-Wallis, χ^2=13.5199, p-value≤0.01). The season with higher FRAP yield for both extracts was summer and it was significantly higher than the seasons with the lowest yields, namely spring and autumn (Dunn test, p-value≤0.05).

The effect of the season over the FRAP values of the EA extract showed statistical differences (Kruskal-Wallis, χ^2=13.0588, p-value≤0.01). The season summer was also the one with the higher FRAP yield and it was significantly higher than the seasons with the lower FRAP values, namely winter and spring (Dunn test, p-value≤0.05).

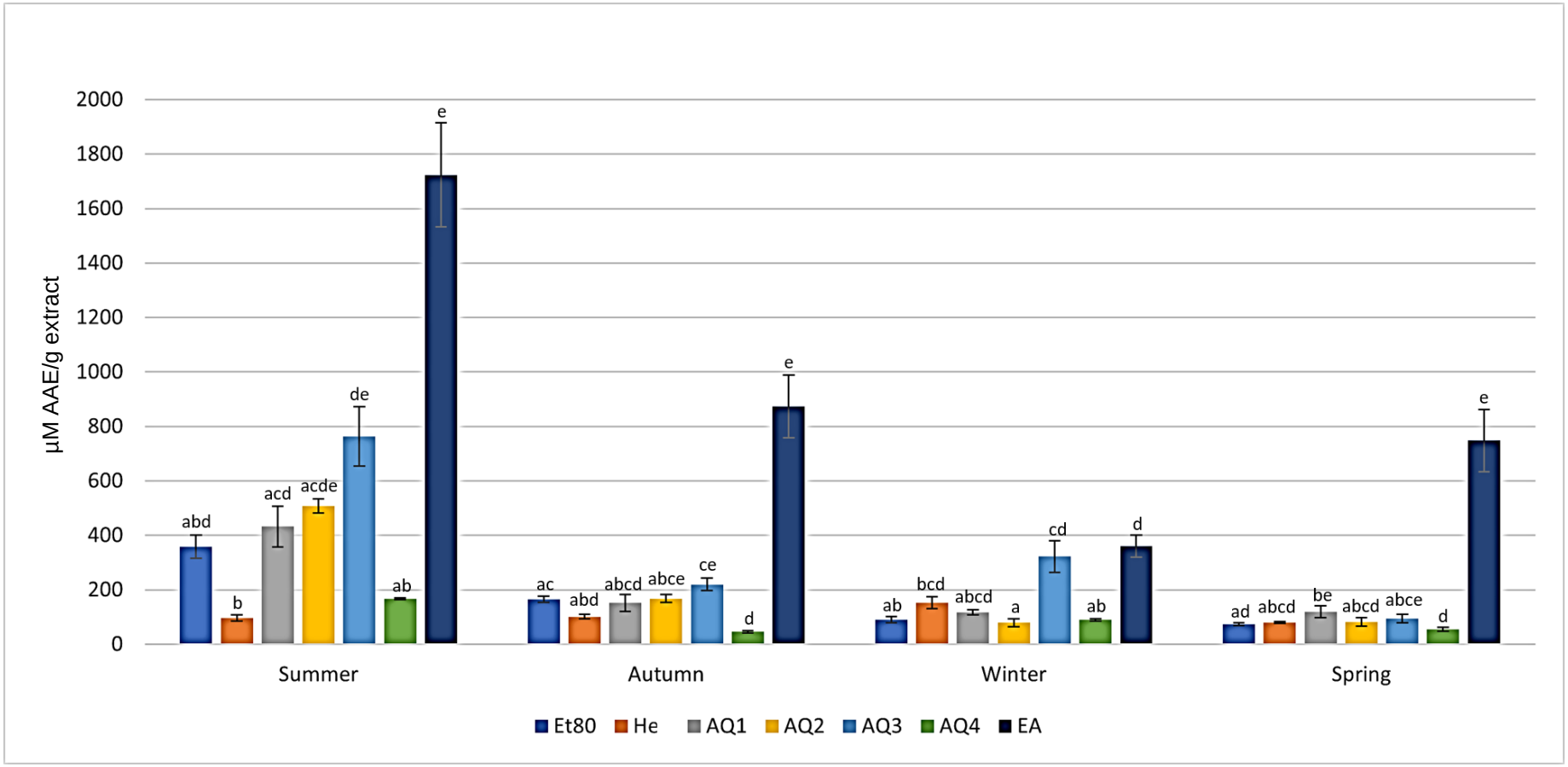

Figure 4.6: Ferric Reducing Antioxidant Power (µM AAE/g extract) of *F. spiralis* extracts for all the seasons. Results are represented as mean ± SD (n=4) and different lowercase letters represent significant differences (Dunn test, p-value ≤ 0.05) between extracts in each season.

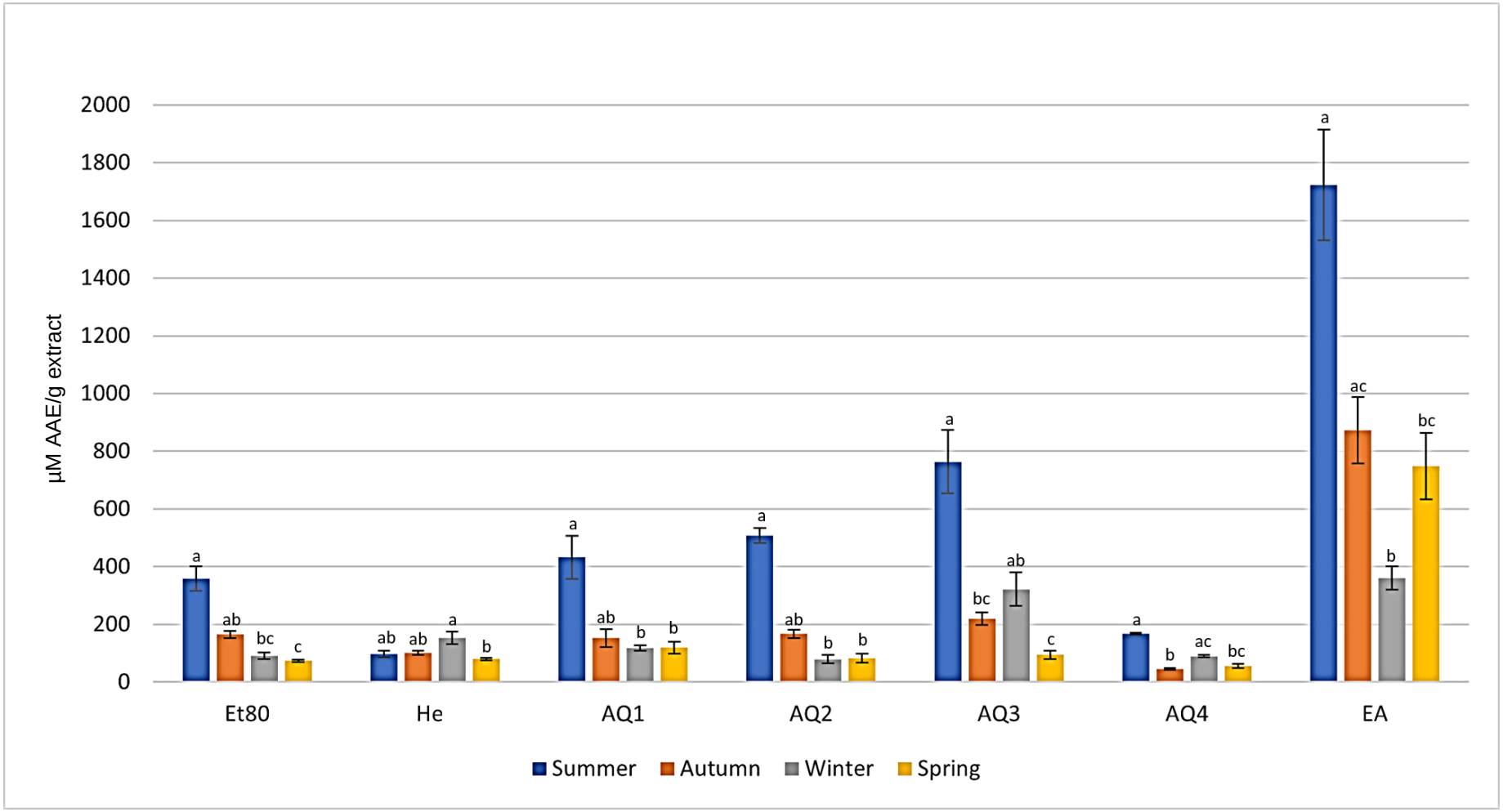

Figure 4.7: Ferric Reducing Antioxidant Power (µM AAE/g extract) of *F. spiralis*, represented for each extract in the four seasons. Results are represented as mean ± SD (n=4) and different lowercase letters represent significant differences (Dunn test, p-value ≤ 0.05) between seasons for each extract.

Previous studies have examined the relation between the antioxidant activity of an algae extract and its phenolic content. Heffernan et al. (2014) found that macroalgae extracts from Atlantic coasts that have previously showed a high polyphenol content, also showed strong ferric reducing antioxidant power. Ethanolic extracts of *F. serratus* exhibited a TPC of 75.96±10.12 µg of GAE while ethanolic extracts of *Laminaria* sp*., Codium* sp*.,* and *Gracillaria* sp*.* Only contained 1.39±0.24, 2.40±0.50, and 4.76±0.17 µg of GAE respectively. When the ferric reducing power of these extracts was analysed, *F. serratus* showed 78.30±15.53 µg of Trolox equivalents while the other three extracts showed 8.51±0.31, 6.01±0.29, and 4.76±0.16 µg TE respectively. Agregán et al. (2018) saw a positive correlation between the TPC and the antioxidant activity of extracts from macro and microalgae, where the ethanolic extract of *F. vesiculosus* that had a TPC of 20 g of phloroglucinol equivalent per 100 g of extract showed a ferric reducing activity of 3.45-3.82 µmol of Trolox equivalent per gram of dry weight, while the ethanolic extract of *Chlorella* sp. contained only 4.5 g of PGE/100 g of extract, and exhibited a relatively low ferric reducing activity of 0.62 µmol TE/g DW. Similarly, the results obtained in this study, showed a relation between the content of polyphenols of the extracts from *F. spiralis* and their ferric reducing antioxidant power. The extracts that demonstrated the higher amounts of phenols in its content, the ethyl acetate extracts from all seasons, also showed the higher antioxidant activity during FRAP testing. Conversely, the aqueous and hexane extracts showed not only a low polyphenolic content but also a low reducing activity.

A difference in the FRAP activity between different extracts of *F. spiralis* has been seen before. Tierney et al. (2013) noticed that if the solvent used for the extraction had yielded high results in TPC, the extract also exhibited a high ferric reducing power. In that study, the ethanolic extract of *F. spiralis* displayed a lower amount of polyphenol content than the methanolic extract, 37.03±3.01 and 39.04±5.72 µg of phloroglucinol equivalent per mg of sample respectively, and the ferric reducing power of the EtOH extract was also lower than the MeOH extract, 20.64±2.19 and 25.63±0.63 µg Trolox equivalent per mg of sample correspondingly.

The correlation between TPC and FRAP seen in this study may be due to the rich content of phlorotannins with a high molecular weight of the ethyl acetate extracts (Hwang et al., 2019; Liu & Gu, 2012), since ethyl acetate has been widely used to selectively extract polyphenolic compounds of intermediate polarity from various vegetal and algal samples, showing effectiveness in enriching phlorotannins from crude extracts (Wang et al., 2012). These phlorotannins present in algae from *Fucus* genus have an antioxidant activity previously associated with their molecular skeleton, since their phenol rings act as electron

traps to scavenge peroxyl, superoxide anions and hydroxyl radicals (Wang et al., 2009), giving them strong antioxidant ability, as seen in this study.

DPPH (1,1-Diphenyl-2-picryl-hydrazyl) free radical assay is based on the scavenging ability of antioxidants towards the reduction of the coloured DPPH free radical. The reduction of colour is proportional to the % reduction of the radical by the antioxidant molecules present in the sample.

The antioxidant activity of several standards was determined using the DPPH method in order to compare with the results obtained for the extracts. The standards had a high percentage of reduction of DPPH, ascorbic acid being the best with a reductive capability of 93.2%, followed by catechol, gallic acid and phloroglucinol with a reductive percentage of 90.43%, 90.35%, and 59.9%, respectively. The standard with the lowest reductive capability was Glucose with 16.8% of reduction of DPPH.

For all the seasons, EA extract was the one that showed the highest DPPH reductive capability with a DPPH reduction percentage of 85.1% for the summer season, 88.1% for the autumn season, 86.1% for the winter season, and 89.4% for the spring season. On the other side, extract AQ4 showed the lowest percentages of DPPH reduction for all seasons, with 21.6% for the summer season, 15.7% for the autumn season, 32.6% for the winter season, and 23.97% for spring season. However, with the exception of the autumn AQ4 extract, the percentages shown by this extract in the remaining seasons are still higher than the reduction % of DPPH displayed by the standard glucose (16.8%).

The results obtained for the effect of the extracts over the reduction percentage of DPPH compound showed statistically significant differences (Kruskal-Wallis, χ^2=62.4436, p-value≤0.01; Fig. 4.8). The EA extract had a higher percentage of reduction of the DPPH compound for every season. These differences were statistically significant for most of the comparisons between extracts (Dunn test, p-value≤0.05; Fig. 4.8).

The effect of the extracts over the reduction percentage of DPPH compound by the extracts from the summer season showed statistical differences (Kruskal-Wallis, χ^2=20.4663, p-value≤0.01). The extract with the higher percentage of reduction of the compound was EA, followed by ET80 and both were significantly higher than the extract with the lowest percentage of reduction for this season, that is AQ4 (Dunn test, p-value≤0.05).

A statistical difference could be seen in the analysis of the effect of the extracts over the DPPH reductive ability of the extracts from the autumn season (Kruskal-Wallis, χ^2 =21.5812, p-value≤0.01). The extract with the highest DPPH reductive ability for this season was EA, followed by extracts AQ3, Et80 and He. Also, all these extracts were significantly higher than the extract with the lowest reduction percentage, namely AQ4 (Dunn test, p-value≤0.05).

The effect of the extracts over the reductive ability of the DPPH compound by the extracts from the winter season also showed statistical differences (Kruskal-Wallis, χ^2=23.9327, p-value≤0.01). The extract with the highest reduction percentage for this season was EA, followed by AQ3, He and AQ2. Also, all these extracts were significantly higher than the extract with the lowest reduction percentage of DPPH compound, that is AQ4 (Dunn test, p-value≤0.05).

The effect of the extracts over the DPPH reductive ability of the extracts from the spring season showed statistical differences (Kruskal-Wallis, χ^2=23.7562, p-value≤0.01). The extract with the highest reduction percentage of the compound for this season is EA, followed by AQ1 and AQ3. In addition, all these extracts were significantly higher than the extract with the lowest DPPH reductive ability, namely AQ4 (Dunn test, p-value≤0.05).

The comparison of the algal extracts to the antioxidant standards was done for each season individually as seen in figures 4.9, 4.10, 4.11 and 4.12.

The results obtained for the effect of the standards and the extracts on the DPPH compound reduction percentage in the summer season showed statistically significant differences. (Kruskal-Wallis, χ^2=27.1334, p-value≤0.01; Fig. 4.9). It was possible to observe that the standards ascorbic and gallic acids, phloroglucinol and catechol had no significant difference among their reductive ability of the DPPH compound whereas the standard with the lowest reductive ability was glucose and was significantly different from the others (Dunn test, p-value≤0.05; Fig. 4.9).

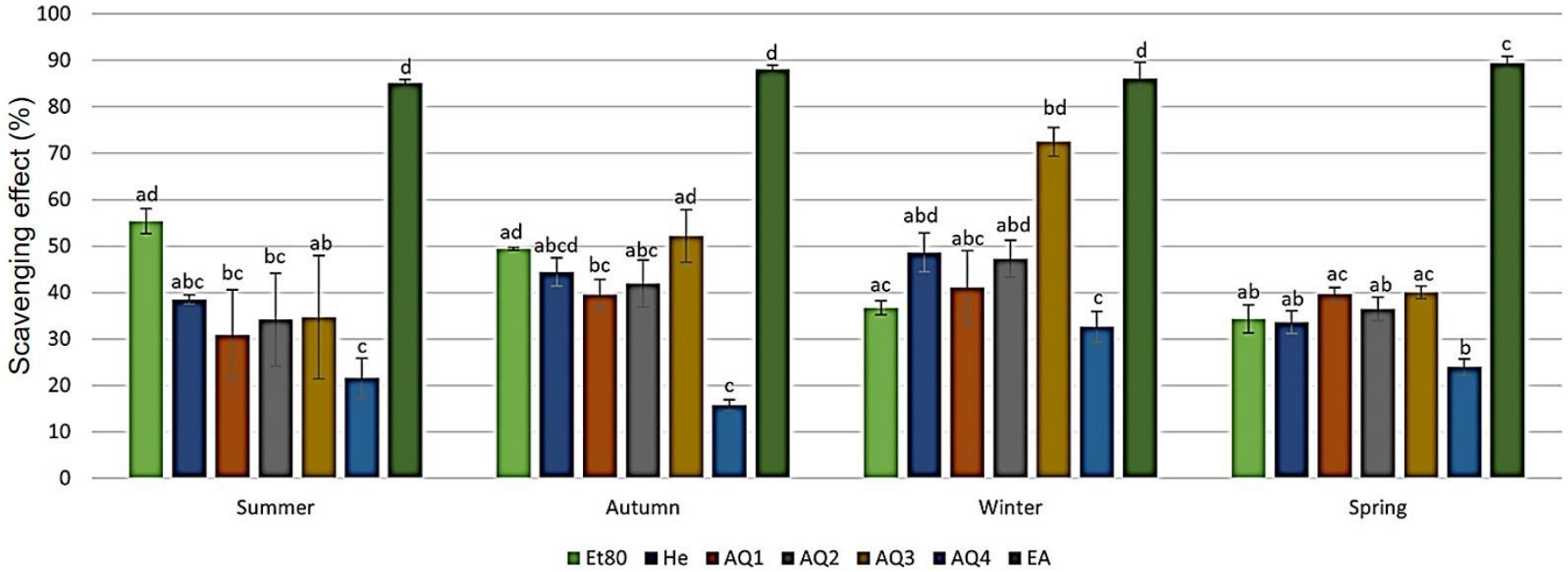

Figure 4.8: DPPH scavenge ability (%) of *F. spiralis* extracts for every season (n=4). Results are represented as mean ± SD (n=4) and different lowercase letters represent significant differences (Dunn test, p-value ≤ 0.05) between extracts of each season.

When comparing the effect of the extracts to the standards over the reduction percentage of DPPH, a statistical difference was observed (Kruskal-Wallis, χ^2=27.1334, p-value≤0.01; Fig. 4.9). The *F. spiralis* extracts AQ1, AQ2, AQ33, and He extract were not significantly different from the standards with the lowest reductive ability of DPPH, glucose and phloroglucinol. On the other hand, the *F. spiralis* extract AQ4 had the lowest reduction percentage and was not significantly different from the standard with the lowest reduction percentage of DPPH, namely glucose. The extracts that showed the highest reduction percentage of DPPH, EA and Et80, showed no significant difference from the standards with the highest reduction percentage, ascorbic and gallic acid, catechol and phloroglucinol, while being significantly higher than the glucose standard (Dunn test, p-value≤0.05).

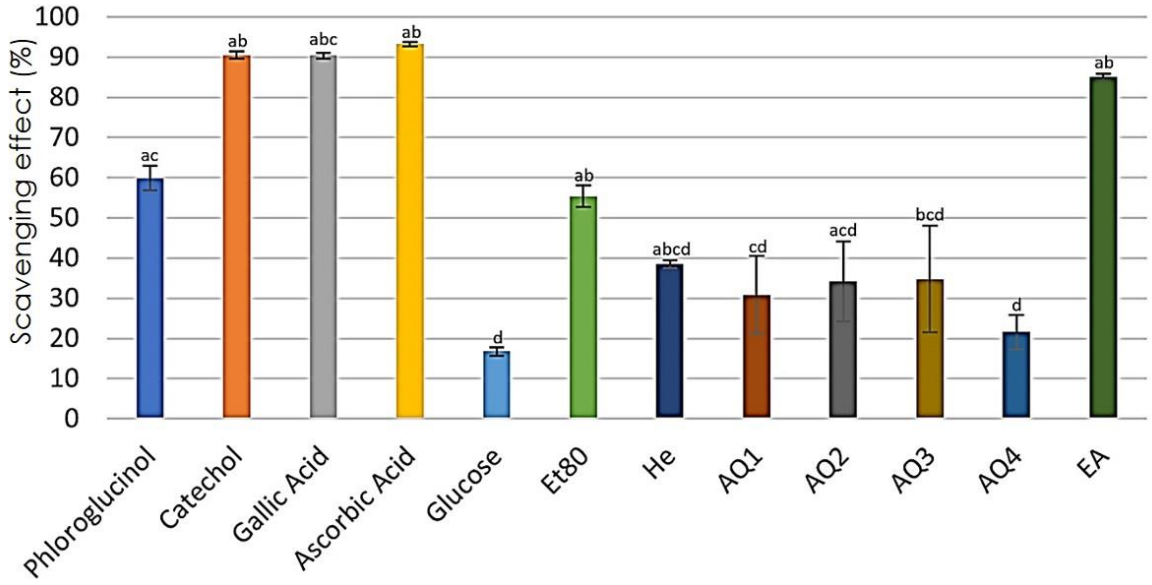

Figure 4.9: DPPH scavenge ability (%) of *F. spiralis* extracts vs. antioxidant standards for the summer season. Results are represented as mean ± SD (n=4) and different lowercase letters represent significant differences (Dunn test, p-value ≤ 0.05) between extracts and standards.

The results obtained for the effect of the standards and the extracts over the reduction percentage of DPPH compound in the autumn season showed statistically significant differences. (Kruskal-Wallis, χ^2=27.5353, p-value≤0.01; Fig. 4.10). It was possible to observe that the ascorbic and gallic acids, the phloroglucinol and catechol standards showed no significant difference among them. On the other hand, catechol and ascorbic acid were significantly different from the glucose standard, which was the standard with the lowest reduction percentage of DPPH and was significantly different from the others (Dunn test, p-value≤0.05; Fig. 4.10).

F. spiralis aqueous extracts AQ1, AQ2, and AQ3 were similar between them. Out of these three extracts, AQ3 was the one that showed the highest reductive ability of the DPPH compound and showed no significant difference with the highly reductive standards gallic and ascorbic acid, phloroglucinol and catechol. The aqueous extract AQ4 showed no significant difference from the glucose standard and was the extract with the lowest reduction percentage (Dunn test, p-value>0.05; Fig. 4.10).

The extract with the highest reductive ability, according to Fig. 4.10, is EA which only showed a significant difference from the lowest reductive standard, glucose (Dunn test, p-value≤0.05), while being similar to the other standards of the test (Dunn test, p-value>0.05).

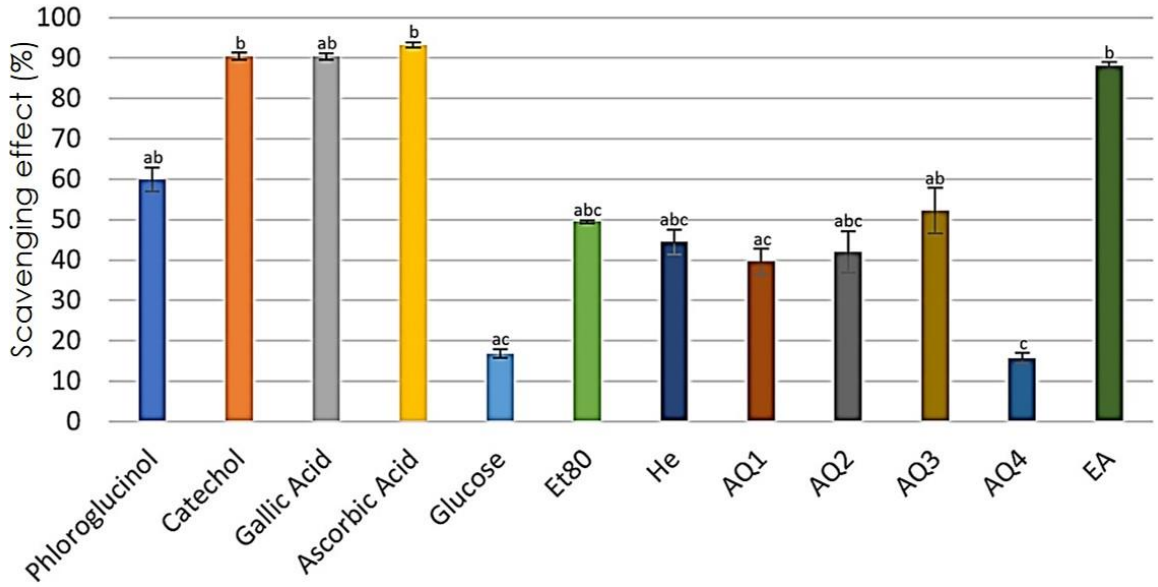

Figure 4.10: DPPH scavenge ability (%) of *F. spiralis* extracts vs. antioxidant standards for the autumn season. Results are represented as mean ± SD (n=4) and different lowercase letters represent significant differences (Dunn test, p-value ≤ 0.05) between extracts and standards.

The results obtained for the effect of the standards and the extracts over the DPPH reduction percentage in the winter season showed statistically significant differences (Kruskal-Wallis, χ^2=29.7804, p-value≤0.01; Fig. 4.11). It was possible to observe that the standards with the higher reduction percentage were ascorbic and gallic acids and catechol, which showed no significant difference among them. Phloroglucinol showed no significant difference to the highly reductive standards and to the lowest reductive standard glucose. Glucose showed a significant difference to the other three standards with high reduction percentage (Dunn test, p-value≤0.05; Fig. 4.11).

The *F. spiralis* aqueous extracts AQ1, AQ2, and AQ4 were not significantly different from the standards with the lowest reductive ability, phloroglucinol and glucose. The aqueous extract AQ3 showed no significant difference to the highly reducing standards, and as seen in Fig. 4.11 this extract was the one with the higher reduction percentage among the aqueous extracts. The Et80 and He extracts showed no significant difference to standards phloroglucinol and glucose. The extract with the highest reductive ability EA showed no significant difference to the standards ascorbic and gallic acid, catechol and phloroglucinol, and it showed a significant difference with the lowest reductive standard, glucose (Dunn test, p-value≤0.05; Fig. 4.11).

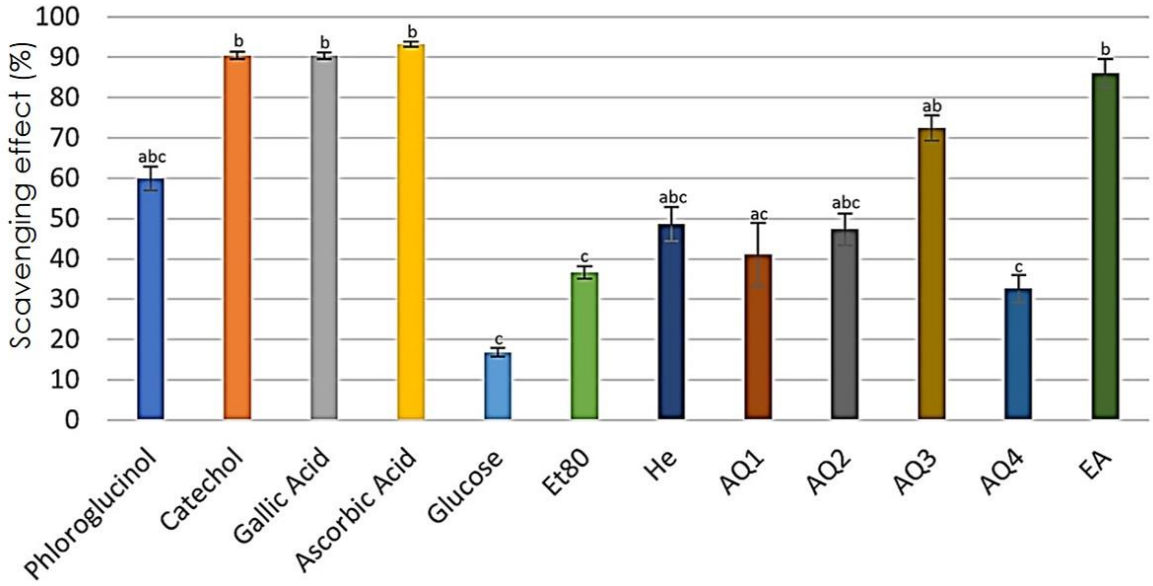

Figure 4.11: DPPH scavenge ability (%) of *F. spiralis* extracts vs. antioxidant standards for the winter season. Results are represented as mean ± SD (n=4) and different lowercase letters represent significant differences (Dunn test, p-value ≤ 0.05) between extracts and standards.

Finally, the results obtained on the effect of the standards and the extracts on the DPPH reduction percentage in the spring season showed statistically significant differences (Kruskal-Wallis, χ^2=29.5561, p-value≤0.01; Fig. 4.12). It was possible to observe that the standards with the highest reduction percentage of DPPH, ascorbic and gallic acids, catechol and phloroglucinol showed no significant difference between them, while the glucose standard had the lowest reduction percentage and it was significantly different to the three standards with the highest reductive ability (Dunn test, p-value≤0.05; Fig. 4.12).

Extracts AQ1 and AQ3 showed no significant difference to any of the standards. Extract AQ2 showed a significant difference to ascorbic acid (Dunn test, p-value≤0.05). As seen in Fig. 4.12 the extract with the lowest reduction percentage was AQ4 and it was significantly lower than every standard except the one with the lowest reductive ability glucose (Dunn test, p-value≤0.05). Et80 and He extracts showed no significant difference from the lowest reductive standards phloroglucinol and glucose. He extract was significantly lower than the three higher reductive standards (Dunn test, p-value≤0.05).

The extract that seemed to have the highest reduction percentage of DPPH, according to Fig. 4.12 is EA, which was significantly higher than the lowest reductive standard glucose (Dunn test, p-value≤0.05). In addition, the result was similar to the standards with the highest reduction percentage ascorbic and gallic acid, catechol, including phloroglucinol (Dunn test, p-value>0.05).

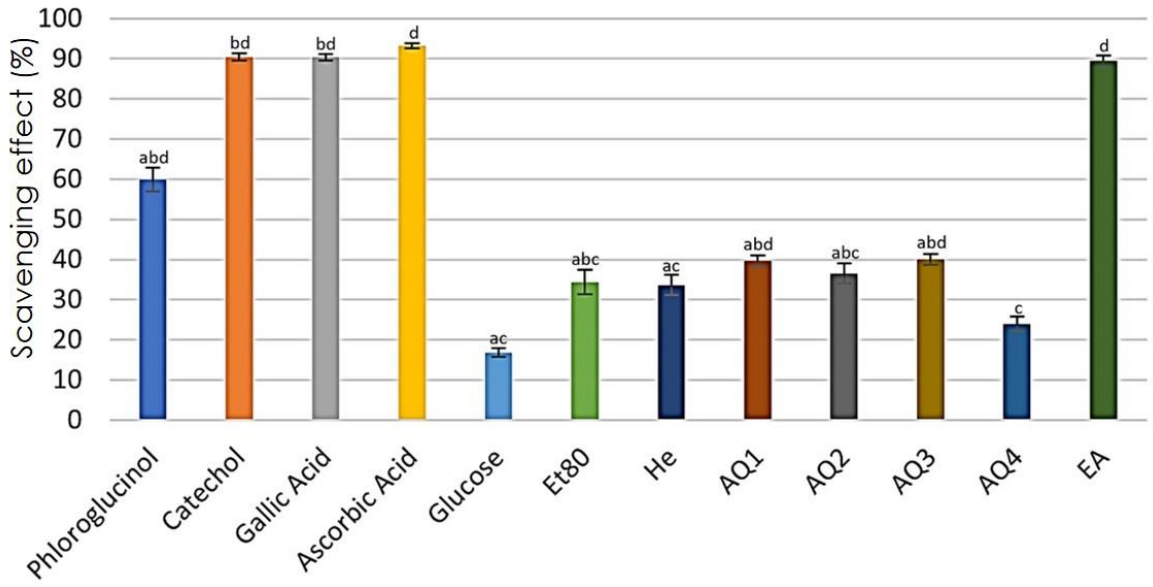

Figure 4.12: DPPH scavenge ability (%) of *F. spiralis* extracts vs. antioxidant standards for the spring season. Results are represented as mean ± SD (n=4) and different lowercase letters represent significant differences (Dunn test, p-value ≤ 0.05) between extracts and standards.

The concentration of the extracts necessary to reduce 50% of the DPPH compound was determined for the extracts of the four seasons that presented higher radical scavenging ability (Et80, EA and AQ3). The IC_{50} for ascorbic acid was also performed for each assay for comparison. The results are shown in Table 4.1 and Figure 4.13.

Table 4.1: IC_{50} of *Fucus spiralis* extracts ET80, EA and AQ3, for all the seasons, in comparison to the IC_{50} of ascorbic acid (AA) standard.

Extract	IC_{50} (µg/mL)			
	Summer	**Autumn**	**Winter**	**Spring**
AA	5.999 (5.195-6.928)	3.881 (3.373-4.465)	3.881 (3.373-4.465)	3.881 (3.373-4.465)
Et80	56.19 (51.11-61.78)	35.24 (31.62-39.28)	61.73 (54.39-70.07)	61.40 (54.69-68.93)
EA	8.414 (7.964-8.890)	4.486 (3.993-5-040)	7.872 (7.215-8.588)	4.450 (4.075-4.860)
AQ3	14.86 (12.93-17.08)	27.92 (25.14-31.01)	12.45 (11.40-13.59)	59.87 (51.94-69.01)

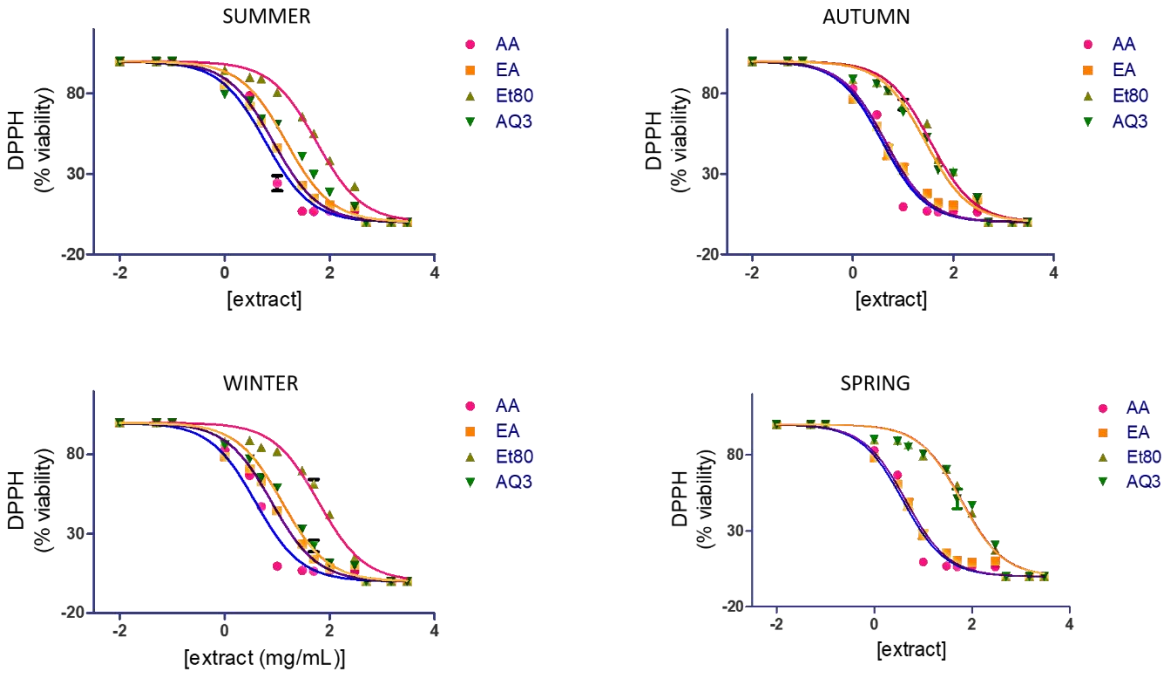

Figure 4.13: IC_{50} of *F. spiralis* extracts Et80, EA and AQ3, for every season, in comparison to ascorbic acid (AA) standard.

For all the seasons, the extract with the lowest concentration necessary to reduce 50% of DPPH was EA extract with a concentration of 8.41 µg/mL for the summer season, 4.49 µg/mL for autumn season, 7.87 µg/mL for the winter season, and 4.45 µg/mL for the spring season. The extract with the highest concentration needed to reduce half of the DPPH compound was Et80 with the following concentrations: 56.19, 35.24, 61.73, 61.40 µg/mL for the summer, autumn, winter, and spring season respectively.

Comparably to the results obtained in the ferric reducing antioxidant power, *F. spiralis* extracts with a high polyphenol content also showed the best scavenging ability. Wang et al. (2012) reported that the ethyl acetate fractions from the ethanolic extract of *F. vesiculosus* exhibited the highest scavenging ability as well as the highest levels of TPC, while the aqueous and n-hexane fractions were less effective and had the lowest phenolic content, as can be seen in this study. Hermund (2016) reported that the ethanolic extract of *F. vesiculosus* had a lower polyphenol content than its ethyl acetate fraction, 20.4±2.4 and 26.5±1.2 g GAE/100 g DW respectively, and that the DPPH radical scavenging ability of the ethyl acetate fraction was also higher than that of the ethanolic fraction. A positive correlation between TPC and DPPH scavenging ability was reported, but it was not proportional, since the TPC of the ethyl acetate fraction was twice as high as the TPC of the water extract, but the scavenging ability was ten times higher. Similarly, the ethyl acetate fraction from *F. spiralis* extract analysed in this study had the higher reducing activity of the DPPH compound for every season. This may be due to the fact that extracts with high radical scavenging ability have also showed high reducing power (Farvin & Jacobsen, 2013).

Polyphenols are well known to show biological and antioxidant activity. Gallic acid is a naturally occurring phenol that is a strong antioxidant and more effective than other soluble antioxidants and its antioxidant activity has often been compared to that of ascorbic acid (Yen et al., 2002). Other antioxidants present in brown algae have demonstrated antioxidant abilities (Catarino et al., 2017). Structurally simple phenols present in brown algae have one hydroxyl group (-OH), catechol and benzenediols have two -OH groups, while phloroglucinol and benzenetriols have three hydroxyl groups. This polyphenolic content is associated with the antioxidant activity of the seaweed (Rajauria et al., 2016). Apart from polyphenols, brown algae also contain sulphated polysaccharides (fucans) that are mainly composed of fucose and sulphate groups, but may also contain amounts of other sugars, like glucose, in their composition. These molecules are of economic importance and have

also demonstrated their potential as free-radical scavengers and antioxidants for the prevention of oxidative damage in living organisms (Rodriguez-Jasso et al., 2014).

In this study, the comparison of the scavenging ability of the extracts of *F. spiralis* was carried out against two strong antioxidant standards (ascorbic and gallic acid), two phenols of increasing complexity (catechol and phloroglucinol) and against a polysaccharide (glucose). Although the difference between these standards was variable among seasons, ascorbic and gallic acid, and catechol had the best results, having more than 90% reduction of the DPPH compound. Phloroglucinol had a lukewarm response, having no more than 60% reduction of DPPH for every season; while glucose showed the lowest reduction percentage of the standards in every season, lower than 20%. It is indicated that the effectiveness and bioactivity of the polyphenols depends on the resonance stabilization of the phenoxy radical, which is influenced by the number of substituents (relative to the hydroxyl group) attached to the aromatic ring at *ortho* and *para* positions. Phlorotannins have complex polymeric structures with up to eight interconnected rings derived from phloroglucinol monomer units, making them the most potent free radical scavenger from polyphenols (Rajauria et al., 2016). As discussed above, the ethyl acetate extracts are phlorotannin-enriched, which would explain why the EA extracts from *F. spiralis* have the highest percentages of reduction of the DPPH compound for all of the seasons, while the aqueous and n-hexane extracts had lower percentages, but no lower than that of glucose. This may be due to the presence of other compounds, such as pigments, proteins or peptides, that may interfere with the scavenging ability of the extracts (Farvin & Jacobsen, 2013), and to the low content of phlorotannins in them. This is in agreement with the results obtained by Li et al. (2017), who analysed the DPPH radical scavenging ability of crude ethanol extracts and its fractions of brown algae, were the IC_{50} of the ethanolic extract was 150,13 µg/mL and the IC_{50} of the ethyl acetate fraction, that was the lowest, was 14,61 µg/mL; while the aqueous extract, had the higher IC_{50} analysed, 206,15 µg/mL. Wang et al. (2012) evaluated the DPPH radical scavenging ability of different solvent fractions of *F. vesiculosus* and compared them with standard antioxidants BHT, α-tocopherol, and ascorbic acid. Ethyl acetate extracts had the higher inhibition percentage in comparison with the hexane, aqueous and n-butanol extracts, similarly to the results presented in our study, were the EA extracts of every season certainly have a higher reduction percentage than the n-hexane and aqueous extracts of *F. spiralis.*

When analysing the IC_{50} of the ethyl acetate and n-butanol extracts and standards, the EA extract had a lower IC_{50} concentration than the latter, 3.76 ± 0.22 and 4.77 ± 0.25 µg/mL respectively. However, the ascorbic acid and BHT standards had a lower IC_{50}, 2.49 ± 0.06

and 3.28 ± 0.09 µg/mL respectively (Wang et al., 2012), comparably to the results of IC_{50} in this study, where EA extracts gave a relatively low concentration when compared to the other extracts of *F. spiralis* analysed, but the standard ascorbic acid had an even lower concentration necessary to scavenge 50% of the DPPH in the samples.

In general, algae are exposed to extreme environmental conditions, dangerous UV radiation, low nutrient availability, salinity, temperature, all of which will induce the formation of oxidizing agents that act as free radicals and other reactive species that can potentially interact with biological systems. Even though, they do not suffer any serious structural and photodynamic damage during metabolism. This resistance may be due to the production of various metabolites, including phenolic compounds, that are known as extremely good reducing agents and free radical scavengers (Mekinić et al., 2019). The biochemical composition of marine algae is known to be highly influenced by geographical location, environment, season, and sampling conditions. Fellah et al. (2017) found that there was a seasonal variation in phenolic compounds content as well as in the antioxidant activity of two species of brown algae analysed similar to the one presented in this study, where the phenolic content of *F. spiralis* extracts was higher for the summer season. This seasonal fluctuation has also been found in other species of the *Fucus* genus. Mendes et al. (2016) found a monthly variation in the phenolic content of *F. vesiculosus*, having the lowest values of TPC for the winter months and the highest during spring, summer and autumn. Paiva et al. (2018) also found seasonal variation in the methanolic and acetone:water extracts of *F. spiralis*, not only in protein, carbohydrate and lipidic composition of the samples, but also in the TPC of the extracts for the summer and winter seasons, obtaining a lower phenolic content in the acetone:water extracts during the winter season, but a lower phenolic content in the methanolic extract for the summer season. This contradiction may be due to the effectiveness of an aqueous organic solution for a better extraction of phlorotannins rather than methanol. However, the difference present in phenolic content between both seasons suggests that the production of phlorotannins by members of the genus *Fucus* is correlated with UV radiation (Paiva et al., 2018), which is in accordance with the results observed in this study, where *F. spiralis* extracts obtained from samples collected during summer had higher values of TPC. This increase in phenolic compound during summer months has been associated with a photoprotective mechanism with dynamic photoinhibition of photosynthesis, in order to tolerate light stress in response to the intensified UV radiation (Mendes et al., 2016).

Since *F. spiralis* is an intertidal species, it consequently is often exposed to high levels of solar radiation over summer, leading to a development of a physiological adaptation, like

the synthesis of a UV-protector, to tolerate this condition of increased radiation and photoperiods. Mancuso et al. (2019) also saw a relation between increasing seawater and air temperatures and the synthesis of phenolic compounds of other intertidal brown algae, finding that the TPC content of algal extracts also increases when the temperature increases above 25 °C, with the higher values found at 28 °C. This hypothesized that the TPC present in this intertidal alga is affected by the thermal seawater conditions experienced during summer and that there are physiological responses to ambient temperature variation. Grazing pressure also seems to affect phenolic content, since their presence deters herbivores (Mancuso et al., 2019). There is a higher incidence of grazing during the summer and early autumn months, when there is less growth and more carbon available for defence chemicals (Mendes et al., 2016).

There also seems to be a relation between phenolic content and the reproductive cycle of the algae. *F. vesiculosus* showed higher contents of phenols during the fertile periods of the summer, and autumn; while showing a lower TPC for winter (Berger et al., 2001). Vandanjon et al. (2017) also found a biochemical variation in brown algae depending on its life cycle, finding a higher percentage of TPC during the months of June and July, during their reproduction stage. In this study, *F. spiralis* also showed the higher polyphenolic content during the summer months, which is the reproductive season of this algae (Coelho et al., 2001).

4.2. Case study: Effect of the incorporation of phlorotannin-enriched extracts in homogenised preparations of fresh salmon during storage

Fish are the main source of polyunsaturated fatty acids that, unfortunately, are highly susceptible to degradation process, such as oxidation (Munekata et al., 2020). The high level of moisture, amino acids and PUFA's, as well as its content in nutrients, autolytic enzymes and high pH turn fish products into highly perishable products that go bad within a short period of time, even when refrigerated (Khalafalla et al., 2015). In order to increase the quality and shelf-life of these products, the use of plant and algal extracts has become a strategy to replace synthetic antioxidants as a source of bioactive compounds.

This case study aimed at evaluating the antioxidant capacity of the phlorotannins-enriched *F. spiralis* extracts to inhibit lipid oxidation in homogenized preparations of fresh salmon. Three different groups of minced salmon patties were prepared: G1-FS, with incorporation of phlorotannins-enriched *F. spiralis* extract (0.01%); G2-BHT, with incorporation of the synthetic antioxidant BHT (0.01%); G3-Ct, without antioxidant incorporation (control). EA extract from summer season was used for incorporation in G2-FS patties, since it was the extract that presented the highest content in phenolic compounds and the higher antioxidant capacity. The salmon patties of the three groups were stored at 4±1 ºC and physicochemical parameters related to fish oxidation were evaluated at days 0, 3, 6, 11, 15 and 21 (Figure 4.14).

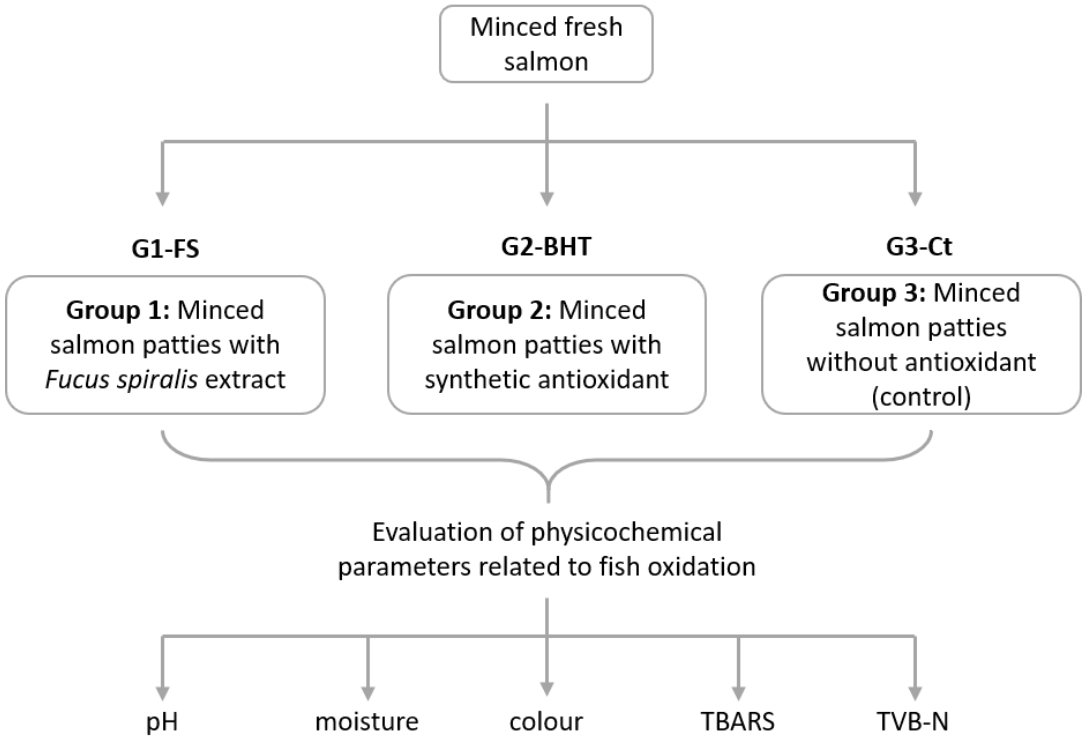

Figure 4.14: Flowchart of the preparation of salmon patties with *F. spiralis* extract and synthetic antioxidant and physicochemical determinations for the evaluation of their degradation.

4.2.1. pH and moisture variations

pH change is an important part in the assessment of the quality of fish products since the pH increase of the fish muscle indicates the accumulation of alkaline compounds, such as ammonia complexes and trimethylamine, which are principally derived from the development and spread of spoilage and pathogenic microorganisms in meat food products (Shokri et al., 2014).

The pH values measured on the surface of the salmon patties of the three groups during 21 days of storage are presented in Table 4.2.

Table 4.2: pH variation of salmon patties over 21 days of storage time. G1-FS, with incorporation of 0.01% *F. spiralis* extract; G2-BHT, with incorporation of 0.01% BHT; G3-Ct, control. The values correspond to the means and standard deviation (n=3). Different capital letters represent significant differences (p-value ≤ 0.05) between treatment groups while different lowercase letters represent significant differences (p-value ≤ 0.05) between the days of storage time.

	G1-FS	G2-BHT	G3-Ct
Day	**pH**	**pH**	**pH**
0	6.283 ± 0.006 ^{Aa}	6.287 ± 0.006 ^{Aab}	6.287 ± 0.006 ^{Aab}
3	6.310 ± 0.010 ^{Ab}	6.307 ± 0.012 ^{Aa}	6.303 ± 0.006 ^{Aa}
6	6.250 ± 0.010 ^{Ac}	6.283 ± 0.006 ^{Bab}	6.263 ± 0.015 ^{ABb}
11	6.260 ± 0.000 ^{Ac}	6.270 ± 0.010 ^{ABb}	6.280 ± 0.010 ^{Bab}
15	6.280 ± 0.000 ^{Aa}	6.277 ± 0.006 ^{Ab}	6.290 ± 0.010 ^{Aab}
21	6.265 ± 0.007 ^{Aac}	6.347 ± 0.040 ^{Aa}	6.340 ± 0.010 ^{Ac}

For the first two days of measurements, there was no statistically significant difference between groups. G1-FS and G2-BHT showed no statistical difference between them, and when comparing both groups to the control group G3-Ct, no statistical difference could be seen either.

On day 6, statistical differences between groups could be seen (Kruskal-Wallis, χ^2=5.333, p-value≤0.05). G1-FS was significantly different from G2-BHT (Dunn test, p-value≤0.05) but when comparing these two groups to the control group (G3-Ct), no statistical differences could be seen.

Statistical differences could be seen between the groups on the eleventh day as well (Kruskal-Wallis, χ^2=5.2099, p-value≤0.05). The treatment groups G1-FS and G2-BHT were not statistically different but when comparing these groups to the control (G3-Ct), only G1-FS was significantly different (Dunett test, p-value≤0.05).

No statistical differences between groups could be seen on the last two days of measurement. This may imply that the treatment of the *F. spiralis* EA extract applied to the patties (G1-FS) as well as the artificial antioxidant BHT (G2-BHT) had no effect on the pH of the samples when compared to the control group after 21 days of storage.

There was also statistical difference in the pH of the treatments throughout the days of testing (Kruskal-Wallis, χ^2=29.657, p-value≤0.01). However, this daily difference in group G1-FS could only be seen among the measurements of the first day and the next three

measurements (Dunn test, p-value≤0.05), followed by similar pH values in the last two days of testing. For the group G2-BHT, the pH was similar the first three measurements but on days 11 and 15 there were slightly lower pH values that were significantly different (Dunn test, p-value≤0.05) from the pH measurement of the last day where the pH was raised again. In the G3-Ct, only the pH measurement of the last day is higher and significantly different from the previous days (Dunn test, p-value≤0.05).

The initial pH presented in the 3 groups of the salmon patties in this study (6.28±0.006) is quite similar to the pH range of fresh salmon mince found in other studies, where a pH of 6.30±0.02 was found in the initial measurements (Khemakhem et al., 2019). A slight increase in the pH can be seen in day 3, where the samples reach a pH value of 6.31±0.01, 6.307±0.012, and 6.303±0.006 for G1-SP, G2-BHT, and G3-Ct, respectively. These values remain within the range found by Khemakhen (2019), however there is a pH decrease on the following measurement (day 6), reaching values of 6.25±0.01, 6.283±0.006, and 6.263±0.015 for each group respectively, followed by an increase in pH in the following days. These results are similar to the ones reported by Wang et al. (2017), where the salmon fillets analysed, showed the same trend in which the values decreased initially and then increased, This may be attributed to the dissolution of carbon dioxide in the fish samples resulting in the decrease in pH, followed by the production of volatile basic components by bacteria that lead to the increase of pH.

Towards the last day of testing, the pH reached values of 6.265±0.007, 6.347±0.04, and 6.34±0.01 for each group correspondingly. These pH values are similar to the ones found by Zamuz et al. (2018), where the meat burgers of the control group presented the same pH (5.52) that the burgers with the antioxidant BHT at the last day of testing; while the burgers treated with the vegetal leaf extracts of chestnut had a lower pH (5.36). This may be due to the acidic nature of the algal extract added to the burgers formulation (Hentati et al., 2019), resulting in a diminishing of pH values. The phlorotannins present in the *F. spiralis* extract may inhibit the degradation of the samples by microorganisms, which leads to a lower production of organic bases and to the stabilization of the patties' pH. This was seen in other studies where the salmon patties treated with antioxidant-enriched olive leaf extracts had a significantly lower pH during shelf-life than the patties of the control group (Khemakhem et al., 2019), and studies where tuna covered with *F. vesiculosus* extracts had a more stable pH during shelf-life when compared to the control group (Vala, 2016).

The average humidity of fish ranges between 66-81%, making it the major component in fish, which is a determinant of the value of products, sensory attributes, and shelf-life in salmon (Dawson et al., 2018). The variation in moisture content of the salmon patties of the three groups along the storage time is presented in Table 4.3. The humidity value was maintained for the whole duration of this study, were the salmon samples had humidity percentages of 62.59±1.328, 62.918±1.03, and 63.016±0.738 for G1-FS, G2-BHT and G3-Ct, respectively, on the first day of testing.

Table 4.3: Moisture content of salmon patties during 21 days of storage. G1-FS, with incorporation of 0.01% *F. spiralis* extract; G2-BHT, with incorporation of 0.01% BHT; G3-Ct, control. The values correspond to the means and standard deviation (n=3). Different capital letters represent significant differences (p-value ≤ 0.025) between treatments while different lowercase letters represent significant differences (p-value ≤ 0.025) between the days of storage time.

	G1-FS	**G2-BHT**	**G3-Ct**
Day	**Humidity (%)**	**Humidity (%)**	**Humidity (%)**
0	62.590 ± 1.328 ^{Aa}	62.918 ± 1.030 ^{Aa}	63.016 ± 0.738 ^{Aa}
3	63.246 ± 0.471 ^{Aa}	63.774 ± 0.586 ^{Aa}	63.055 ± 0.308 ^{Aa}
6	62.675 ± 0.779 ^{Aa}	63.588 ± 0.668 ^{Aa}	62.538 ± 0.408 ^{Aab}
11	62.727 ± 0.441 ^{Aa}	64.303 ± 0.728 ^{Aa}	64.075 ± 0.296 ^{Aa}
15	61.829 ± 0.183 ^{Aa}	62.314 ± 0.186 ^{Aa}	61.200 ± 1.212 ^{Ab}
21	62.945 ± 0.597 ^{Aa}	63.716 ± 0.478 ^{Aa}	62.742 ± 0.229 ^{Aab}

No statistical difference between the humidity of the groups throughout the time of measurement could be seen between G1-FS and G2-BHT, or when comparing these groups to the control (G3-Ct).

Treatment G1-FS has a humidity that averages 0.77% lower than G2-BHT, and 0.10% higher than G3-Ct, which may indicate that *F. spiralis* ethyl acetate extract may not be as good as the artificial antioxidant BHT to avoid water loss during shelf-life but is slightly better than the control group. This trend was also seen by Vala (2016), where tuna fillets covered with gelatine containing algal extracts with high levels of antioxidants have humidity percentages somewhat higher than the control samples. However, this slight difference can be attributed to the heterogeneity of the samples, and these punctual variations may be due to sample variability (Khemakhem et al., 2019).

Loss of humidity in fish muscle happens due to liberation of exudate during shelf-life. This water loss of fish products causes the loss of its organoleptic properties, making it important to keep the humidity of the samples as stable as possible during storage (Vala, 2016), or at least between the average humidity found on fish. This could be observed during this study, where even at the last day of testing, the humidity reached values between the average, with percentages of 62.945±0.597, 63.716±0.478, and 62.742±0.229 for G1-FS, G2-BHT and G3-Ct, respectively.

4.2.2. Lipid oxidation analysis

Ammonia has proven to be an excellent indicator of the quality of sea fish, making the dosage of total volatile basic amines (TVB-N) an essential characteristic in the assessment of the quality of seafood products. It also allows to describe the advanced stages of spoilage in fish (Kabamba, 2016), since it is an indicator of the presence of nitrogenous materials resulting from the action of proteolytic bacteria (Soares et al., 2013).

Knowing that the increase of TVB-N content in fish flesh reflects the spoilage degree of this product, the more the fish is expose to open air, the more the putrefaction increases (Kabamba, 2016), ergo, a variation and increase of volatile bases through time is expected.

The total volatile basic amines content of the salmon patties samples from all of the groups for days 0, 3, 6, 11, 15, and 21, can be seen in Figure 4.15.

The initial values of TVB-N found in this study for the control group (13.691±0.087 mg/100 g) are similar to the values found by Mei et al. (2020) in the initial measure of total volatile nitrogen bases of fresh Atlantic salmon fillets (13.65 mg/100 g), indicating freshness of the samples used.

Statistical differences in the content of ammoniac and volatile amines throughout the duration of the test were observed (Kruskal-Wallis, χ^2, =37.7596, p-value≤0.05). As can be seen in the figure 4.15, the volatile amines content increased towards the last day of the test and was significantly higher than the volatile amines content of the first days (Dunn test, p-value≤0.05).

These results are similar to the ones found by Sur et al. (2016), where a significant increase of the total volatile basic nitrogen content in the flesh of another species of salt water fish (*Sparus aurata*) was found during the storage in ice during 12 days of storage, increasing almost three times from day 0. Kabamba (2016) also obtained TVB-N values on fish's flesh

that rose from 29.47±1.82, and 48.37±2.43 mg/100 g of sample on the first day of testing, to 51.67±1.45, and 50.31±1.84 mg/100 g of sample on the last day respectively.

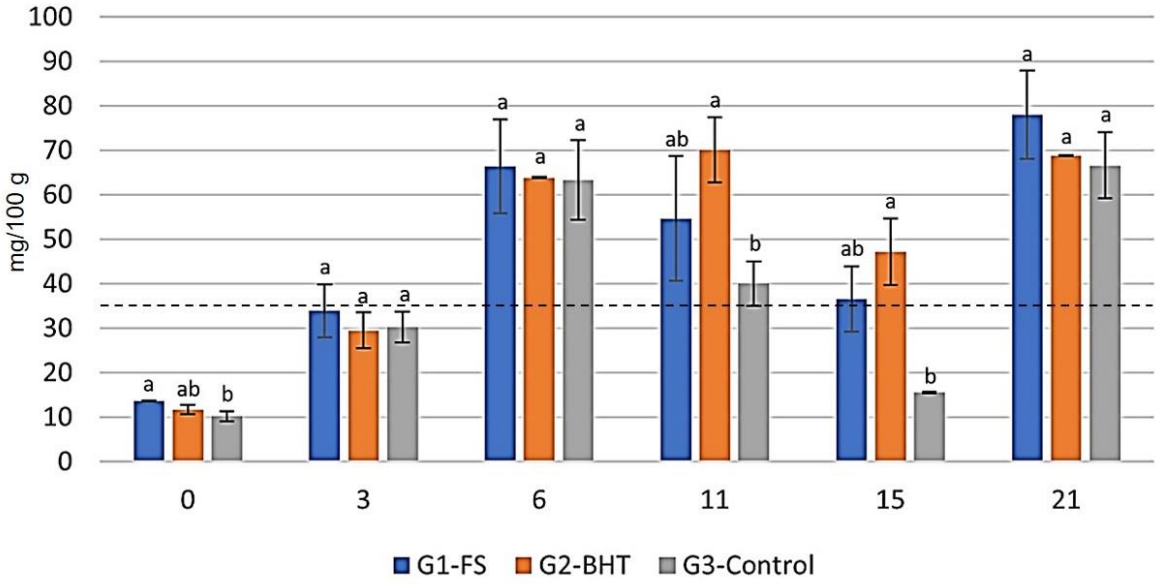

Figure 4.15: TVB-N (mg/100 g of sample) of salmon patties during 21 days of storage. Treated with *F. spiralis* extract (G1-FS), BHT (G2-BHT), and control (G3-Ct). The values correspond to the means ± standard deviation (n=3). Different lowercase letters represent significant differences (Dunn Test, p-value ≤ 0.05) between groups for each day of storage time. The dotted line represents the legal limit stipulated for Atlantic salmon ((EC) No 1022/2008).

The decrease of the nitrogen volatile bases in G1-FS and G3-Ct for day 11 and 15, and G2-BHT on day 15, may be due to measurement errors. In the last day of measurement, an increase in the TVB-N values, where G1-FS has a slightly higher content of nitrogen volatile bases than G2-BHT and G3-Ct (78.058±9.884, 68.833±0.115, and 66.627±7.414 mg/100 g respectively) can be seen.

In the present study, when analysing the effect of the treatments over the volatile bases' content of the salmon samples in the first day of testing, a statistical difference was observed (Kruskal-Wallis, χ^2=5.3611, p-value ≤ 0.05). No significant difference between G1-FS and G2-BHT could be seen. However, G1-FS was significantly higher than the control group (G3-Ct) (Dunett test, p-value ≤ 0.05).

No statistical difference between the volatile bases content of the salmon samples on days 3 and 6 could be seen between G1-FS and G2-BHT or when comparing these groups to the control (G3-Ct).

When analysing the effect of the treatments over the content of volatile bases of the salmon samples in days 11 and 15, a statistical difference was observed (Kruskal-Wallis, χ^2=4.4643, p-value ≤ 0.05). No significant difference between G1-FS and G2-BHT could be seen. However, G2-BHT was significantly higher than the control group (G3-Ct) (Dunett test, p-value ≤ 0.05).

No statistical difference between the volatile bases content of the salmon samples on the last day of testing could be seen between G1-FS and G2-BHT, or when comparing these groups to the control (G3-Ct).

The lack of significant difference between all the groups on the last day of measurement means that the algal and artificial antioxidant did not have a significant effect in the production of volatile bases in the salmon patties when compared to the control treatment. These values are not similar to the trend found by Besbes et al. (2017), who found a significant difference in the content of volatile base nitrogen of seafood treated with polyphenol-enriched extracts versus the control group (22.12±0.29 and 32.07±0.22 mg/100 g, respectively). Even though, they are comparable to the trend found by Soares (2013), who had a stable nitrogen content for the treatments and control, during 14 weeks of storage under 5 °C for salmon with antioxidant-enriched covers. The authors argue that the storage time may not have been enough to observe differences among the different treatments over the spoilage bacteria and the fact that low temperatures during storage can slow down the activity of endogenous enzymes. That may explain the trend in the TVB-N values showed in the present study, values that mirrored the ones found by Soares in not exceeding the legal limit (35 mg/100 g of muscle) stipulated for Atlantic salmon until day 3, according to the regulation (EC) No 1022/2008 (European Union, 2008).

Thiobarbituric acid reactive substances (TBARS) assay is a method to detect lipid oxidation. It measures malonaldehyde (MDA), which is a product of the oxidation of unsaturated fatty acids. MDA reacts with thiobarbituric acid (TBA) to form a pink chromogen (Khalafalla et al., 2015). Changes in TBARS values of fresh salmon patties during storage at 4±1 ºC are presented in Figure 4.16.

The initial values of malonaldehyde found in this study are 0.446±0.0624, 0.8718±0.1483, and 1.2733±0.2178 mg MA/Kg sample for G1-FS, G2-BHT, and G3-Ct respectively. These quantities are higher than those found by Rizo et al. (2015) in fresh salmon, who found no evidence of malonaldehyde in his samples, and Baek et al. (2019) that obtained 0.55±0.02 mg MA/Kg sample of salmon on the first day of measurement. This discrepancy may be

due to the difference in freshness of the salmon used in the present study, meaning that there may have been a degradation of polyunsaturated fatty acids previous to the first measurement.

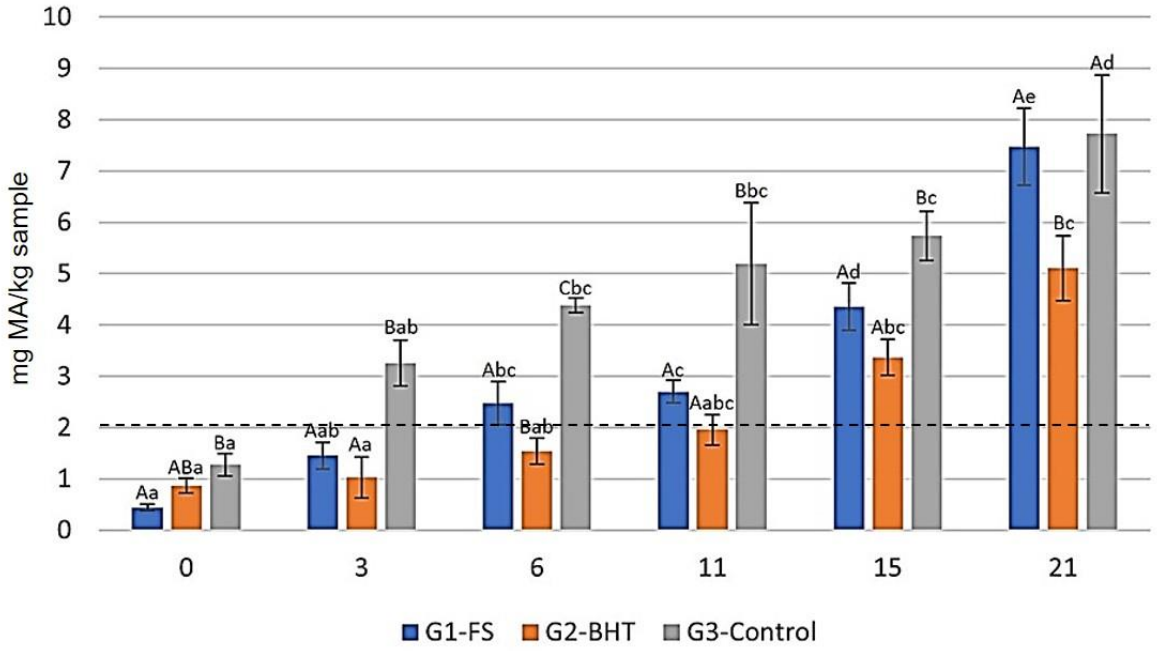

Figure 4.16: TBARS (mg MA/Kg sample) values of salmon patties during 21 days of storage (measured at days 0, 3, 6, 11, 15 and 21). Treated with *F. spiralis* extract (G1-FS), BHT (G2-BHT), and control (G3-Ct). The values correspond to the means ± standard deviation (n=3). Different capital letters represent significant differences (p-value ≤ 0.05) between the treatment groups while different lowercase letters represent significant differences (p-value ≤ 0.05) between the days of storage time. Dotted line represents limits for the development of undesirable odours (Connel, 1990).

There is statistical difference when analysing the effect of the time over the amount of malonaldehyde from the products of every group when the testing was performed (Kruskal-Wallis, χ^2=38.5677, p-value≤0.01).

For G1-FS and G3-Ct the measurement of the last day is significantly higher and different from the measurements of every other previous day for both groups (Dunn test, p-value≤0.05). For G2-BHT the last measurement shows no difference from the previous two days. There also seems to be a similarity between two consecutive measurements in the samples of the groups, meaning that the amount of malonaldehyde produced from the oxidation process of the fish patties is not so large as to be significantly different from one

day of measurement to the next, but the production seems to increase as time passes by, showing a significant production of this decomposition product in the last days of testing (Fig. 4.16).

This constant increase of malonaldehyde is comparable to the increase seen in previous studies performed by Khemakhem et al. (2019) in salmon treated with vegetal extracts throughout the storage period of 18 days, where the TBARS values of the control group and treatments rose from 0 mg MA/Kg sample to an approximate range of 0.10-0.25 mg MA/Kg sample. The same was observed by Albertos et al. (2019) in fresh fish patties coated with antioxidant-enriched seaweed films stored at 4 °C for 7 days, where the level of MA increased over storage in every treatment, as well as the control.

On the last day of measurement, the TBARS values reached 7.4728±0.7433, 5.1055±0.6286, 7.7246±1.1473 mg MA/Kg sample for G1, G2 and G3 respectively, indicating that the samples with the artificial antioxidant BHT were the least oxidized of the three. These results coincide with the study carried out by Yarnpakdee et al. (2019) on the antioxidant activity of macroalgae extracts against lipid oxidation of tuna slices, where after 10 days of storage, the control samples, as well as the treatments with algae extracts, had a higher content of malonaldehyde than the sample with the artificial antioxidant BHA.

When analysing the effect of the treatments over the amount of malonaldehyde of the samples over the time of study, a statistical difference could be seen (Kruskal-Wallis, χ^2=8.6543, p-value≤0.01). Only on day 6, a significant difference between G1-FS and G2-BHT (Dunn test, p-value≤0.05) and when comparing these groups to the control G3-Ct (Dunett test, p-value≤0.05) could be seen. No statistical difference could be seen between the groups with natural (G1-FS) and artificial antioxidant (G2-BHT) on measurement days 0, 3, 11 and 15. Both these groups also showed significantly lower amounts of malonaldehyde when compared to the control (G3-Ct) (Dunett test, p-value≤0.05) meaning that the *F. spiralis* extract added to the salmon patties, had a similar effect to the artificial antioxidant BHT in the conservation of the product until storage day 15, lowering the content of deterioration products for the first days of the study when compared to the control group.

On day 21, the TBARS content of the samples from G1-FS was significantly higher than the content of the samples from G2-BHT (Dunn test, p-value≤0.05). Since the patties of G2-BHT also showed a malonaldehyde content that was significantly lower than the content from the control G3-Ct (Dunett test, p-value≤0.05), the artificial antioxidant had a positive effect on the conservation of the product when compared to the control group and to the salmon patties with the algal extract.

These results may reflect a dependency of the antioxidant's ability, artificial or of algal origin, to inhibit lipid oxidation to its concentration on the samples, situation previously observed by Merlo et al. (2019) on the effects on the shelf-life of salmon fillets covered in films with antioxidant-enriched extracts, where even when the polyphenolic extracts had previously shown high antioxidant activity, it did not increase the antioxidant ability of the films over the degradation of salmon. This indicated that the protection against oxidation could depend on the type of antioxidant found in plant species and also on their concentrations, making the establishment of TBARS values a difficult task since many internal and external factors can influence it. Miraglia et al. (2016) also analysed different concentrations of phenolic extract on the quality of salmon steak during storage, where at the end of the shelf-life of the samples analysed, he observed that the ones treated with the highest phenolic concentration showed the lowest TBARS levels, implying that perhaps a higher concentration of phenolic extract may result in a better conservation of the product.

4.2.3. Colour variation

Surface colour and appearance are important factors for consumers when purchasing salmon (Wang et al., 2017). Since fish appearance and colour originate from muscle/water binding properties and pigmentation within the skin or meat surface, these can be oxidized resulting in darkening or fading. Salmon meat has a pink pigmentation in its natural state, and after storage and freezing the colour tends to fade (Dawson et al., 2018). The major influence of food colours on the consumer's acceptance, colour indices (L*, a* and b*) can be seen in Table 4.4. The value L* represents the overall lightness of the sample, while a* signifies the redness or greenness and b* signifies the yellowness of the sample of the salmon patties with algal treatment (*F. spiralis* extract 0.01%), artificial treatment (BHT 0.01%), and control group for days 0, 3, 6, 11, 15, and 21.

Table 4.4: Colour parameters L*, a*, and b* of salmon patties during 21 days of storage. Treated with *F. spiralis* extract (G1-FS), BHT (G2-BHT), and control (G3-Ct). The values correspond to the means and the standard deviation (n=9). Different lowercase letters represent significant differences (p-value ≤ 0.05) between the days of storage time. Since there is no significant difference between groups it is not represented on the table.

Treatment		Day					
		0	3	6	11	15	21
G1	L* (D65)	65.19 ± 1.28 [a]	65.54 ± 1.91 [a]	64.71 ± 1.69 [a]	65.61 ± 0.99 [a]	65.02 ± 0.40 [a]	65.60 ± 2.89 [a]
	a*	14.96 ± 0.23 [a]	14.79 ± 0.29 [ab]	13.49 ± 0.30 [bc]	13.26 ± 1.04 [cd]	12.17 ± 0.56 [de]	11.89 ± 0.43 [e]
	b*	23.77 ± 0.68 [a]	25.49 ± 0.51 [b]	23.46 ± 0.63 [ab]	24.11 ± 2.01 [a]	22.99 ± 0.32 [a]	23.50 ± 1.48 [a]
G2	L* (D65)	64.29 ± 0.68 [a]	64.09 ± 1.98 [a]	65.67 ± 0.79 [a]	64.40 ± 0.93 [a]	64.58 ± 1.09 [a]	64.21 ± 1.31 [a]
	a*	15.26 ± 0.21 [a]	14.76 ± 0.55 [ab]	14.71 ± 0.28 [bc]	13.75 ± 0.27 [cd]	13.07 ± 0.45 [de]	12.62 ± 0.32 [e]
	b*	23.66 ± 0.53 [a]	24.88 ± 1.42 [b]	25.35 ± 0.40 [ab]	23.74 ± 0.51 [a]	23.54 ± 0.72 [a]	22.94 ± 0.76 [a]
G3	L* (D65)	63.63 ± 1.54 [a]	65.18 ± 0.45 [a]	64.38 ± 0.90 [a]	65.03 ± 0.26 [a]	63.92 ± 0.27 [a]	64.67 ± 1.25 [a]
	a*	15.07 ± 0.26 [a]	14.63 ± 0.22 [ab]	13.61 ± 0.32 [bc]	12.94 ± 0.42 [cd]	12.32 ± 0.16 [de]	11.15 ± 0.64 [e]
	b*	22.98 ± 0.34 [a]	25.04 ± 0.39 [b]	24.16 ± 0.50 [ab]	23.74 ± 0.50 [a]	23.73 ± 0.07 [a]	22.82 ± 0.19 [a]

When analysing the effect of the treatments over the lightness of the samples (L*), no statistical difference could be seen between the groups. There was no significant difference between the groups with antioxidant (G1-FS and G2-BHT), and between these groups and the control (G3-Ct) throughout the time of the study.

When analysing the effect of time over the lightness of the samples (L*) for every group, no statistical difference could be seen between the days of measurement There was no significant difference between the days of measurement.

The results observed by Khemakhem et al. (2019) when measuring L* value of salmon patties treated with plant extracts, showed that the storage time had no significant effect on lightness in any type of salmon patty, nevertheless, unlike our study, the incorporation of the extracts by Khemakhem increased the lightness of the samples (slightly higher L* value of treated samples vs. control). The changes in the value L* are related to changes in the muscle surface light scattering. Structural changes of the muscle proteins brought upon by post-mortem decay increase the light scatter of the surface region of the salmon patty over time, indicating an opaque colour (Merlo et al., 2019). The stability of L* value throughout the duration of this study may indicate that the cold storage conditions were able to prevent sample discolouration or to observe a difference between treatments (Khemakhem et al., 2019).

When analysing the effect of the treatments over the redness of the samples (a*), no statistical difference could be seen between the groups. There was no significant difference between the groups with antioxidant (G1-FS and G2-BHT) and between these groups and the control (G3-Ct) throughout the time of the study, meaning that the colour red was not affected by any of the antioxidants, if any, used.

When analysing the effect of time over the redness of the samples (a*) for every group, a statistical difference could be seen between the days of measurement (Kruskal-Wallis, χ^2= 44.0949, p-value≤0.01). There was significant difference between the initial day of measurement when compared to posterior days, 6, 11, 15, and 21 (Dunn test, p-value≤0.05), meaning that the red colour of the samples was affected by the variable of time, having decreased significantly towards the last days of testing.

In a study with fish patties fortified with algae extracts, Hentati et al. (2019) observed a change in colour after the addition of the algae, lowering the a* values of the patties with algae when compared to the control group. This slight decrease in redness of the samples, also observed in the present study, may be due to the fact that the *Fucus* extract has brown pigments typical from brown algae that may interfere with the measurement of colour. A

similar situation was observed by Khemakhem et al. (2019), where the addition of an extract rich in brown pigments to salmon patties led to a decrease in the redness of the samples. Nevertheless, these studies also showed an increase in a* value throughout storage time, unlike the decreasing trend observed in the present study, which may suggest that *F. spiralis* extract added to the formulation did not impede the discolouration of the samples via lipid oxidation products as aldehydes and ketones (Zamuz et al., 2018). This decrease was also observed in the study carried out by Vala (2016), where a significant descent in the a* value could be seen in fish samples covered with antioxidant-enriched gelatine as well as in the control groups, implying that the loss of red tone of the salmon samples over the time of the study is related not only to lipid oxidation, but also to the oxidation of myoglobin, converting oxymyoglobin, of a bright red colour, to metmyoglobin, of brown colour.

When analysing the effect of the treatments over the greenness of the samples (b*), no statistical difference could be seen between the groups. There was no significant difference between the groups with antioxidant (G1-FS and G2-BHT), and between these groups and the control (G3-Ct) throughout the time of the study, meaning that the colour green was not affected by any of the antioxidants, if any, used.

When analysing the effect of time over the greenness of the samples (b*) for every group, a statistical difference could be seen between the days of measurement (Kruskal-Wallis, χ^2= 22.266, p-value≤0.01). There was a slight increase of the value on day 3 for all of the groups, showing a significant difference between this day of measurement when compared to posterior days, 6, 11, 15, and 21 (Dunn test, p-value≤0.05). However, the value b* decreases again and was more or less stable the following days until the last day.

This stability in the colour parameter b* was seen by Khemakhem et al. (2019), where no effect of storage time was observed for parameters a* and b*, and by Hammond & Skonberg (2012), who found no significant effects of individual treatments, or concentration of the antioxidant used on yellowness (b*) on Atlantic salmon fillets during storage time.

5. Conclusions

The present study revealed a variability in the phenolic content, as well as the antioxidant properties relative to the different extracts obtained from the extraction and subsequent fractionation of *Fucus spiralis*. The EA extracts from this alga showed the higher total polyphenolic content and as expected, the iron chelating activity (FRAP) and DPPH radical scavenging ability were significantly higher in these extracts when compared to the others, showing that the extraction of enriched algal extracts using adequate solvents can increase the antioxidant activity of the samples. Seasonal variations can also be observed in the content of phenolic compounds as well as the antioxidant activities of *F. spiralis* extracts, since biotic and abiotic factors may affect the production and concentration of antioxidant compounds in brown algae. The extracts obtained from *F. spiralis* collected during the summer season showed the higher total phenolic content out of every season and, since relatively high levels of phenolic constituents are linked to more potent antioxidant activities, these extracts demonstrated the best iron chelating and DPPH scavenging abilities as well. The biochemical composition observed, as well as the high functional bioactivity values showed by the summer EA extract provided information on the best extract and period of the year to collect this seaweed for its potential use as a natural antioxidant for fish derived products.

Patties prepared with salmon flesh and treated with 0.01% EA extract of *F. spiralis* from the summer season showed a stable pH and humidity percentage throughout storage time, similarly to the patties treated with 0.01% BHT and the control group. No significant difference in the nitrogen volatile bases content or on the analysed colour parameters was observed between the groups. However, the extract had a similar behavior to the artificial antioxidant BHT regarding the diminishing of deterioration compounds (decrease of thiobarbituric acid reactive species TBARs) on the first days of measurement (until day 11) when compared to the control. Even though marine algae extracts that have shown high amounts of phenolic components have also shown an ability to decelerate or prevent oxidation processes, the summer EA extract of *F. spiralis* that showed the higher TPC content, accompanied by the stronger antioxidant abilities, did not particularly improved the functional properties of the salmon patties during storage.

Consequently, further investigation is still necessary in order to use the extracts obtained from the evaluated macroalgae as possible fortifier agents in fish-based products and as an effective alternative to the synthetic chemical supplements commonly used, that may cause undesirable effects on consumers. There is a need to optimize the formulation needed to,

not only increase the oxidative stability of the products, but also, to not affect their organoleptic quality or their nutritional value, so as to obtain functional ingredients with specific health benefits that will potentially improve consumers' quality of life.

6. References

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